Studies on the expression and function of p56Lck and Core Binding Factor during thymocyte development

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Abstract

Haematopoiesis, the generation of mature blood cells, is controlled by signals that activate lineage specific genes, allowing for cellular proliferation, survival and differentiation. This study focuses on the regulation and function of genes coding for molecules specifically involved in T cell development. The first gene investigated was the gene coding for the lymphoid-specific, Src-family protein tyrosine kinase, p56\textsuperscript{Lck} that was first discovered as being over-expressed in a lymphoma cell line. This kinase has been shown to play a vital role in thymocyte differentiation. The timing of p56\textsuperscript{Lck} expression during thymocyte development was determined. The results show that the expression of the p56\textsuperscript{Lck} gene is developmentally regulated at the post-transcriptional level at the precise stage at which its upstream receptor complex, the pre-TCR, is expressed. In addition, transgenic mice expressing green fluorescent protein (GFP) under the control of the p56\textsuperscript{Lck} proximal promoter were generated and analysed. The implications of these results for future use of this promoter are discussed.

Secondly, the role of Core Binding Factor (CBF), a heterodimeric transcription factor consisting of an \(\alpha\) and a \(\beta\) subunit, in thymocyte development was investigated. Translocations targeting the CBF\(\alpha2\) and CBF\(\beta\) genes are amongst the most frequent mutations in human leukaemia. The \(\alpha\) subunits contain a conserved DNA binding RUNT domain, via which these CBF proteins bind to sequences in a variety of T cell specific genes. CBF\(\alpha2\) and CBF\(\beta\) deficient mice have been generated but their early lethality precludes the study of T cell differentiation in these mice. CBF dominant negative transgenic mice were generated in order to investigate the role of these transcription factors in thymocyte development. Preliminary analysis of these mice has revealed a potential role for CBF in the regulation of thymocyte survival.
Acknowledgements

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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukaemia</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>βMEC</td>
<td>beta-mercaptoethanol</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-[cyclohexylamino]-1-propane sulfonic acid</td>
</tr>
<tr>
<td>CBF</td>
<td>core binding factor</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CIP</td>
<td>calf alkaline phosphatase</td>
</tr>
<tr>
<td>CLP</td>
<td>common lymphoid progenitor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>causes recombination</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5’-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine 5’-triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine 5’-triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine 5’-triphosphate</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>double negative CD4-CD8- thymocytes</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNA-PKC</td>
<td>DNA-dependent protein kinase</td>
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<td>DN4</td>
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</tr>
<tr>
<td>DP</td>
<td>double positive CD4+CD8+ thymocytes</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>E</td>
<td>embryonic day</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>diaminoethanetetra acetic acid</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FCS</td>
<td>foetal calf serum</td>
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<td>Abbreviation</td>
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<tr>
<td>FISH</td>
<td>fluorescence in situ hybridisation</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>hGH</td>
<td>human growth hormone</td>
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<tr>
<td>HSC</td>
<td>haematopoietic stem cell</td>
</tr>
<tr>
<td>IRE</td>
<td>iron response element</td>
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<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>ISP</td>
<td>immature single positive</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoglobulin family tyrosine-based activation motif</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>lauria broth</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>LoxP</td>
<td>locus of crossover (x) in PI bacteriophage</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MoMuLV</td>
<td>Moloney murine leukaemia virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-Morpholino]propane sulfonic acid</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
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<tr>
<td>NK</td>
<td>natural killer</td>
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<td>NP40</td>
<td>Nonidet P-40, r-tert-octylphenyl 9.6 ethoxylate</td>
</tr>
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<td>OD</td>
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<td>OLB</td>
<td>oligonucleotide labelling buffer</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood lymphocyte</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>pTa</td>
<td>pre-TCRa chain</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
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<tr>
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<td>position effect variegation</td>
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<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
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<tr>
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</tr>
<tr>
<td>RAG</td>
<td>recombinase activating gene</td>
</tr>
<tr>
<td>RCN</td>
<td>relative copy number</td>
</tr>
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</table>
RSSs recombination signals
RNA ribonucleic acid
rpm revolutions per minute
RT reverse transcriptase
RT-PCR reverse-transcriptase polymerase chain reaction
SB saponin buffer
SC RT-PCR Single cell reverse-transcriptase polymerase chain reaction
SCID severe combined immunodeficiency
SDS sodium dodecyl sulphate
SH Src-homology domain
SP single positive thymocytes, either CD4+CD8- or CD4-CD8+
tRNA transfer RNA
TAE tris/ acetic acid/ EDTA buffer
TCR T cell receptor
TEMED N,N,N,N,N-tetramethylethylenediamine
Th helper T cell
Tm melting temperature
TNT Tris/ sodium/ Tween-20
Tris tris(hydroxymethyl)aminomethane
Tween-20 polyoxyethylenesorbitan monoluarate
UV ultra violet
w/v with respect to volume
5'UTR 5' untranslated region
Chapter 1

General Introduction

1.1. Haematopoiesis

Haematopoiesis is the process whereby mature blood cells of distinct lineages, including lymphocytes, are produced from pluripotent haematopoietic stem cells (HSCs). For this lifelong process to be maintained HSCs must have the capacity of self-renewal and also the capacity to differentiate into cells of multiple lineages. Within the mammalian embryo haematopoiesis occurs in two phases in different locations. The initial stage of haematopoiesis is termed 'primitive' and occurs within the yolk sac blood islands, resulting in cells of the primitive erythroid lineages. All other lineages are formed during the second phase of definitive haematopoiesis that occurs within the foetal liver, spleen and finally the bone marrow (for review see Keller et al., 1999). Definitive haematopoiesis therefore results in the formation of all cells of myeloid, lymphoid and erythroid lineages that form the components of the blood system (see Figure 1.1). Of these, the cells that develop from the myeloid and the lymphoid progenitors into cells of the immune system will be discussed here.

The immune system consists of a wide range of cell types, including neutrophils and macrophages which represent part of the innate, non-specific arm of the immune system, and T and B lymphocytes which form the basis of the adaptive arm of the immune system. These lymphocytes play a major role in the immune response since they possess on their surface receptors specific to antigens presented by pathogens. The highly variable antigen receptors of T and B cells, the T cell receptor (TCR) and B cell receptor (BCR) respectively, are generated during development via site-specific recombination of germline genes and allow for the recognition of an enormous range of invasive pathogens.
In the foetus, B cells develop in the liver and in the bone marrow, and during adult life exclusively in the bone marrow. These cells recognise specific antigen via surface immunoglobulin that forms the BCR. Antigen binding to these immunoglobulin receptors initiates B cell activation, resulting in the clonal expansion of the B cell and differentiation into an antibody secreting plasma cell. B cells recognise unmodified proteins and mainly provide defence against pathogens or their products in extracellular spaces. T cell precursors generated in the foetal liver or bone marrow colonise the thymus where they develop into naive CD4+CD8- or CD4-CD8+ single positive T cells. The TCR consists of an antigen binding heterodimer of polymorphic immunoglobulin-like αβ or γδ chains which bind antigen, in association with invariant proteins of the CD3 complex which are responsible for intracellular signalling. αβT cells recognise their targets by detecting peptides fragments derived from the foreign protein and bound to specialised cell surface molecules called major histocompatibility complex (MHC) proteins on the infected cell. These MHC molecules are of two types, class I and class II. Mature αβ T cells are also of two effector types, cytotoxic CD8 single positive T cells and helper/inflammatory CD4 single positive T cells. MHC class I molecules bind to peptides from proteins synthesised within the cytosol, such as viral proteins and are recognised by the cytotoxic T cells, whereas MHC class II molecules bind to proteins in intracellular membrane-bound vesicles and can be recognised by helper T cells.
1.2. The Thymus

The majority of thymus-dependent lymphocytes develop in the thymus from precursors originating from other haematopoietic tissues. Work using thymectomised mice in the 1960s established the central role of this organ in thymocyte development (Miller 1961). Certain populations of T cells however do not develop in the thymus, especially those found between the epithelial cells of the gut mucosa. These will not be discussed here (for a review of extrathymic T cell differentiation see Rocha et al., 1995).

In addition to thymocytes, other cell types require the thymus for development. These include a separate lineage of cells termed Natural Killer (NK) T cells that pass through the thymus during their development. Precursors to these cells give rise to T cells within the thymus, whereas if removed from the thymus and intravenously transferred into an irradiated host animal this population generates functional NK cells (Rodewald et al., 1992). This finding and the fact that there are numerous phenotypic similarities between NK cells and T cells led to the search for a common progenitor cell (Lanier et al., 1992). Investigations into the developmental potential of human CD34+CD3-CD4-CD8- cells via clonal cell differentiation assays has since led to the identification of a common T/NK progenitor (Sanchez et al., 1994). These cells express low levels of an αβTCR and CD3 can be distinguished by staining with anti-NK1.1 in appropriate mouse strains. Note that γδ NK T cells have also been reported (for review see Fehling et al., 1999). Thymic dendritic cells (DCs) also develop within the thymus from an endogenous precursor (Ardavin et al., 1993) which as mentioned below has the capacity alternatively to differentiate into B or T cells. It is thought that interactions between developing T cells and these newly formed thymic DCs allow for the deletion of cells reactive to intrathymic self antigens (Ardavin et al., 1993). B cell progenitors are also present in the thymus. A recent study has shown that these thymic B cell progenitors can develop into mature B cells with distinct characteristics to peripheral B cells when injected intrathymically into an allotype-disparate recipient (Mori et al., 1997). In this study, no mature B cells developing from thymic B cell progenitors were seen to migrate to the periphery. However in another study, utilising intrathymic injections of FITC (fluorescein isothiocyanate), thymic emigrants stained with
FITC contained mature B cells as well as αβ and γδ T cells. A significant number (approximately $2 \times 10^6$) of B220+IgM+ mature B cells develop in the thymus and are exported to the periphery in C57BL/6 mice (K. Akashi unpublished data, see Akashi et al., 2000).

1.2.1. The Thymic environment

The cellular compartmentalisation and stromal cell content of the thymus provide a perfect microenvironment for the development of mature T cells. It is well established that the interactions between stromal cells and thymocytes are crucial in cell-lineage decisions and in T cell development. The thymic stroma is complex, being composed of epithelial cells, mesenchymal cells, macrophages, DCs and matrix molecules, and provides both the cell-cell interactions and soluble factors required for thymocyte maturation. The thymic environment can be viewed as being equally complex as the T cell differentiation it regulates (see Boyd et al., 1993 for review; Anderson et al., 1996). Contact with thymic epithelial cells is vital for all stages of thymocyte development. Nude mice, which lack a normal thymus due to stromal defects, fail to support T cell development (Jordan et al., 1977). Different thymic stromal compartments are required at different stages of development (Anderson et al., 1993a). The mesenchymal (fibroblast cells) and MHC class II+ epithelial cells are both required for the development of day 14 embryonic thymic precursors into mature T cells, whereas MHC class II+ epithelial cells alone can support later stages of development. This work went on to show that although a murine fibroblast cell line 3T3 could act as a substitute for foetal mesenchyme when combined with purified epithelial cells, the supernatants produced by these 3T3 cells were not able to support the maturation of day 14 precursors. This provides the first evidence that the early stages of thymocyte development are dependent on direct interaction with mesenchymal fibroblasts.

1.2.2. The role of Growth Factors in Lymphocyte Development

Stromal cell types produce a variety of soluble factors or cytokines including interleukin 7 (IL-7) and stem cell factor (SCF). The precise role of these cytokines in commitment, differentiation and survival is not fully understood but the generation of mice deficient in certain cytokines has elucidated a vital
role for these growth factors in haematopoietic development (see Figure 1.2). For example, mice deficient for the gamma chain of the Interleukin 2 receptor (IL-2Rγ) completely lack NK cells and are impaired in development of immature T and B cells (DiSanto et al., 1995). The IL-2Rγ chain is a common component of receptors for IL-2, IL-4, IL-7 and IL-15, suggesting that one or more of these cytokines are involved in lymphoid development. A specific role for IL-7 in lymphoid development is established since mice deficient for IL-7 are lymphopenic, with thymic cellularity reduced twenty-fold and splenic cellularity reduced ten-fold (von Freeden-Jeffry et al., 1995). Mice deficient for the interleukin 7 receptor α chain have also been generated (Peschon et al., 1994; Maki et al., 1996). All mice showed a dramatic reduction in cellularity with the thymus reduced to 0.1 - 10% and the spleen to 10% of wild-type values. Peschon reported a variation in phenotypes with 66% of mice arrested early in thymocyte development at the CD4-CD8- stage, while 33% did show differentiation to the CD4+CD8+, CD4+CD8- and CD4-CD8+ stages. γδ T cells are completely lacking in these mice while NK cells were present in normal numbers. In addition, stem cell factor (SCF, otherwise known as c-kit ligand) also has a role in T cell development in mice, with deficiency resulting in a forty-fold reduction in thymic cellularity (Rodewald et al., 1995). Mice deficient for both SCF and the IL-2Rγ chain have also been analysed (c-kit-γc-) (Rodewald et al., 1997). Mice lacking both of these growth factor receptors show a very severe, early thymocyte-specific developmental arrest. In fact, by cell counting not even CD4-CD8- DN thymocytes were detected within the thymus of these mice. B cells do develop in these mice. Interestingly thymic DCs develop in these mice despite the lymphoid phenotype, so revealing that thymic DCs can develop independently of thymocytes. The thymic stroma was not properly organised into distinct medullary and cortical areas and was infiltrated with connective tissues. This implies that cell to cell contacts that normally occur within the thymic stroma are prevented in the absence of these two receptors and also that alternative thymocyte growth factors cannot compensate for the lack of c-kit and the IL-2Rγ chain at this early stage in thymocyte development. The thymic environment, the cells it is composed of and the growth factors they produce are therefore vital for thymocyte development.
1.2.3. Lymphoid progenitors cells

Intrathymic development of thymocytes begins with stage-specific homing of foetal liver or bone marrow stem cells to the thymus. The extent of T cell commitment prior to arriving in the thymus is not clear. As discussed above, progenitor cells with the potential to develop into T or NK cells have been
identified within the thymus (Sanchez et al., 1994), as have precursors able to differentiate into T, B or DC cells (Ardavin et al., 1993). Early precursors with the potential to give rise to T cells, B cells, NK cells, and dendritic cells have also been identified in the mouse thymus (Wu et al., 1991a; Matsuzaki et al., 1993). The thymus is therefore seeded with multipotent HSCs or with separate lineage committed stem cells or with both (for review see Shortman and Wu 1996). Recently a clonogenic common lymphoid progenitor (CLP) has been identified that can differentiate into T, B and NK lineages but not into myeloid lineages (Kondo et al., 1997). It has been discussed that the commitment of CLPs to either, say the T or B lineage, may be determined by the microenvironment that CLPs encounter; those reaching the thymus may mainly differentiate into T cells, whereas other CLPs differentiate into B cells in the bone marrow. Alternatively Enver and Greaves proposed that multipotent progenitors maintain their plasticity for differentiation into alternative lineages through expression of lineage-related genes at a low level and lineage fate is determined through amplifying the expression of certain lineage-associated transcription factors (Enver and Greaves 1998). The transcription factors identified thus far as important in T cell lineage commitment will be discussed later.

In addition to the above mentioned transcription factors, genes of the Notch family of transmembrane receptors are also involved in control of cell fate decisions during thymocyte development. Notch proteins are highly conserved transmembrane receptors that regulate cell fate choices throughout development of many cell lineages (Artavanis-Tsakonas et al., 1999). The role of Notch signalling in the generation of cell diversity, via the cell-cell interaction process of lateral inhibition, is well documented. A number of recent reviews cover the role of Notch signalling in multiple steps in T cell development so this will be kept brief here (von Boehmer 1999; Deftos and Bevan 2000). Notch activity is generally regulated by binding of the Notch receptor to ligands of the Jagged/Delta family, which result in cleavage of the receptor such that the intracellular domain, known as Notch IC, is released. Notch IC is then transported to the nucleus where it acts as a transcription factor in a complex with the DNA-binding protein CBF1/RBP-Jk. Downstream targets of Notch
signalling include genes encoding the Hairy Enhancer of Split (Hes) transcription factors which act to inhibit the expression of tissue-specific bHLH transcription factors (Jarriault et al., 1995; Kageyama and Ohtsuka 1999). A role for Notch 1 in the specification of cell fate was first suggested upon the analysis of Notch 1-deficient T cells (Radtke et al., 1999). Conditional inactivation of the Notch gene in thymocytes, using the interferon-inducible Mx-Cre transgenic system, resulted in an arrest in thymocyte development at or before the most immature DN1 stage. B cell, erythroid and myeloid development in these mice was unaffected so demonstrating a role for Notch signalling in specification of T cell fate.

The control of precursor homing to the thymus is just beginning to be understood. Extracellular matrix proteins, chemoattractants, integrins and CD44 (Pgp1) have all been implicated (Dunon and Imhof 1993; Savino et al., 1993). Studies utilising transgenic mice expressing green fluorescent protein (GFP) under the chicken-β-actin promoter have addressed this question of adhesion and migration of mouse foetal liver cells to the thymus (Kawakami et al., 1999). Foetal liver cells from GFP transgenic mice (previously generated Okabe et al., 1997) were incubated with deoxyguanosine-treated embryonic day 14 thymic lobes and the migration of GFP positive cells into the lobes was observed. The involvement of the extracellular matrix receptor CD44 in precursor homing to the thymus was first investigated. CD44 binds to hyaluronic acid and to a lesser extent, collagen. Anti-CD44 antibody treatment at the beginning of the repopulation period resulted in GFP positive thymocytes adhering to the surface of the thymus lobe but not migrating into the thymus as seen without anti-CD44 treatment. In addition, treatment with anti-integrin α4 antibodies inhibited even the adhesion stage. These data suggest that integrin α4 is required for the adhesion of foetal liver cells to the thymus lobe and CD44 is required for the migration of the cells into the thymus.

1.2.4. Thymocyte development

T cells can be divided into two subsets based on the type of T cell receptor (TCR) they express. In the adult the majority of T cells express a TCR consisting of an α and a β chain, the remaining cells express an alternative TCR made of γ and δ chains. The status of the TCR can be used, in conjunction with
the presence of cell surface markers such as CD4, CD8, CD25 and CD44 to follow the phenotypic differentiation of αβ T cells (see Figure 1.3). Gene targeting studies and the generation of transgenic mice have been very useful tools in further dissecting the stages of thymocyte development. Within the adult thymus T cell precursor cells have been identified that are low expressors of CD4, Thy1 and heat-stable antigen (HSA) and high expressors of CD44 and c-kit (stem cell factor receptor) (Wu et al., 1991b; Godfrey et al., 1994). These T cell precursors are present in low numbers constituting only 0.2% of all thymocytes. The T cell receptor of these cells is in germline configuration (i.e. is unrearranged) and they can develop along the αβ or γδ lineage. These cells are not yet fully committed to the T cell pathway. Although they can no longer give rise to myeloid or erythroid cells they can still develop into B cells, NK cells and thymic DCs (Ardavin et al., 1993).

The first stage of maturation can be identified as the upregulation of Thy1 and HSA and the loss of CD4 expression. This heterogeneous population of cells comprising 2-4% of thymocytes is termed double negative (DN) CD4-CD8- and can be further subdivided based on the combinatorial expression of CD44 and CD25. The first population, from now on referred to as double negative population 1 (DN1) are therefore CD44+c-kit+ and CD25- (the interleukin 2-receptor α chain). The upregulation of CD25 expression results in CD44+c-kit+CD25+cells of the second DN population (DN2). This transition is accompanied by the first phase of proliferation within the DN subsets (Penit et al., 1995). These cells can no longer give rise to B cells but can still develop along the pathway towards thymic DCs (Wu et al., 1996). It is within this population that rearrangement of the TCR chain genes commences, resulting in the detection of D to Jβ gene transcripts within these DN2 cells (Godfrey et al., 1993; Wilson and MacDonald 1995). The loss of CD44 and c-kit expression leads to CD44-c-kit-CD25+ cells (DN3). This transition is accompanied by a number of vital events in thymocyte maturation and so this DN3 stage is of much developmental interest. The DN3 to DN4 represents the first major checkpoint in T cell development. Within these DN3 cells rearrangement of the TCRβ loci is completed resulting in the production of full-length TCRβ transcripts (Godfrey et al., 1993). These cells are now fully committed to the T cell lineage and can no
longer generate thymic DCs (Wu et al., 1996). Those cells that have produced a functional rearranged TCRβ chain are selected and undergo a process known as β selection, expand and develop into the final subset of the double negative compartment via down regulation of CD25 expression (CD44-c-kit-CD25-DN4). Successful TCRβ rearrangement also results in the phenomenon of allelic exclusion by which rearrangement at the second TCRβ locus is shut down.

Further studies of the DN3 stage have resulted in the discovery that this population is heterogeneous and may be split into different sub-populations based on their pre or post β-selection status (Hoffman et al., 1996). 90% of these cells are non-dividing cells of expected size (termed 'E' cells). These are described as being prior to selection. The remaining 10% of these cells are large (termed 'L' cells), with features of cycling cells such as increased DNA content, increased expression of cyclin A and B, and increased activity of cdc2 (cell-division-cycle kinase 2) and CDK2 (cyclin-dependent kinase 2). L cells have mostly in-frame TCRβ rearrangements and are described as being post-β-selection. Therefore, it can be seen that the β selection is associated with events that ensure thymocyte proliferation as the cells transit to the fourth and final subset of the double negative compartment. It is at this DN3 to DN4 transition that mice deficient for recombination activator genes (RAG 1 and 2) are blocked in thymocyte development (Mombaerts et al., 1992b; Shinkai et al., 1992). RAG 1 and 2 initiate V(D)J rearrangement of the TCRβ chain by cleaving DNA at conserved recombination signals (RSSs) that flank all antigen-receptor variable region gene segments, generating coding and RSS ends. RSS ends are then precisely joined, whereas coding ends are joined in a process that can involve nucleotide gain or loss upon ligation (see Schatz 1997 for review). Thymocytes from these mice are unable to rearrange their TCRβ chains and so cannot progress beyond this point. A naturally occurring mutation results in severe combined immunodeficient (SCID) mice that are also blocked in thymocyte development at this stage. These mice cannot efficiently rearrange their TCR or immunoglobulin genes due to a mutation in a gene encoding for a DNA-dependent protein kinase that is required for recombining the gene segments (Blunt et al., 1995; Kirchgessner et al., 1995). Gene targeting strategies have been used to investigate further the role of components of the DNA-end-joining
Figure 1.3: Thymocyte development

Import from foetal liver/bone marrow

<table>
<thead>
<tr>
<th>Thymic lymphoid progenitors</th>
<th>DN1</th>
<th>Thymic lymphoid progenitor</th>
<th>Multipotent, limited self-renewal</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit+CD44+CD25-CD4lo in the adult</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro T cell</td>
<td>DN2</td>
<td>TCRβ,γ and δ</td>
<td>rearrangement onset</td>
</tr>
<tr>
<td>C-kit+CD44+CD25+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early pre-T cell</td>
<td>DN3</td>
<td>Unproductive TCR-β rearrangement</td>
<td>β selection</td>
</tr>
<tr>
<td>c-kit-CD44-CD25+</td>
<td></td>
<td></td>
<td>P56lck-mediated signal</td>
</tr>
<tr>
<td>Late pre-T cell</td>
<td>DN4</td>
<td>Unproductive TCR-α rearrangement</td>
<td>TCRα rearrangement onset</td>
</tr>
<tr>
<td>C-kit-CD44-CD25-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+/lo CD8+CD3-</td>
<td>ISP</td>
<td>Unproductive TCR-α rearrangement</td>
<td>negative selection/failed positive selection</td>
</tr>
<tr>
<td>Double positive cells</td>
<td>DP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+CD8+CD3 k/kit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single positive cells</td>
<td>SP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+CD8-CD3+CD4-CD8+CD3+</td>
<td></td>
<td></td>
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<td>Export to periphery</td>
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</table>

reactions used for repairing double strand breaks in DNA and for V(D)J rearrangement of the TCRβ chain. These components include DNA-dependent protein kinases (DNA-PKCs), the Ku heterodimer (Ku70-Ku80) and a factor called XRCC4 that interacts with DNA ligase IV (for review see Smider and Chu 1997). These studies have revealed that mice lacking Ku70 or Ku-80 are immunodeficient due to being unable to rejoin coding or RSS ends (Nussenzweig et al., 1996; Ouyang et al., 1997) whereas DNA-PKCs deficient mice are immunodeficient due to defects in rejoining coding ends but not RSS
ends (Gao et al., 1998). Mice lacking DNA ligase IV (except for being embryonic lethal) show the same characteristics as Ku70 or Ku80 deficient mice again due to impaired V(D)J rearrangement (Frank et al., 1998). In mice deficient for the TCRβ chain there is an incomplete block in thymocyte differentiation at the DN to DP transition, with a significantly reduced number of DP thymocytes developing (Mombaerts et al., 1992a). Additional studies with these deficient mice have further underpinned the crucial role of TCRβ gene rearrangement or expression in the development of DP cells. The introduction of a fully rearranged, functional TCRβ chain transgene into these RAG deficient or SCID mice rescues thymocyte development resulting in the production of a high number of DP cells (Shinkai et al., 1993; Shores et al., 1993). Mice deficient for both the TCRβ and the TCRδ chain are completely blocked at the DN stage.

These late DN4 cells can progress to the CD4+CD8+ double positive (DP) stage via intermediates that express either CD4 or CD8 without a mature αβ TCR and are so called immature single positives (ISPs). The transition to the DP stage also involves TCRα chain rearrangement and expression (Nikolic-Zugic and Moore 1989). Cells that successfully rearrange the TCRα chain proliferate and rapidly develop into CD4+CD8+ DP cells that express the αβ TCR-CD3 complex. Mice deficient for the TCRα chain develop normally to the DP stage but are mostly devoid of SP cells (Mombaerts et al., 1992a; Philpott et al., 1992). At this stage these DP cells undergo the processes of positive and negative selection based on the reactivity of the αβ TCR-CD3 complexes with MHC molecules (see section 1.4.1). A low percentage of DP cells further mature into single positive CD4+CD8- or CD4-CD8+ SP cells. This differentiation from DP to SP is the final important checkpoint in thymocyte development.

1.3. The role of the pre-TCR in thymocyte development

The stages of thymocyte development have been briefly described above. However, there are two main developmental checkpoints within thymocyte differentiation that need further discussion. These are the progression from DN to DP stage and from the DP to SP stage. These two points are regulated by the surface expression of the pre-TCR and the mature αβ TCR respectively.
As discussed, the rearrangement and expression of the TCRβ chain at the DN3 stage is vital for further development from this point. How can the TCRβ chain promote T cell development without a functional TCRα (this gene is not rearranged until the DP stage)? Via investigations of the TCRβ-CD3 complex or the 'pre-TCR' on the surface of TCRβ-transfected immature T cells from SCID mice with this question was answered. The pre-TCR was found to contain a novel 33kDa type 1 transmembrane protein covalently attached to the TCRβ and CD3 components. This protein, termed the pre-TCRα or pTa is a non-rearranging member of the immunoglobulin superfamily (Groettrup et al., 1993; Saint-Ruf et al., 1994). Analysis of the expression of the pTa has shown it to be restricted to immature thymocytes, being absent from B cells, NK cells, myeloid cells and mature αβ T cells (Bruno et al., 1995). The pre-TCR is expressed at a level 100 times lower than the TCR on mature T cells and it has been difficult to detect by flow cytometric analysis. Recent work using a sensitive immunofluorescent technique has however allowed the surface expression pattern of the pre-TCR to be analysed (Bruno et al., 1999). Within the DN thymocyte subsets the pre-TCR is detected on the surface of a proportion of DN3 cells and on the majority of DN4 cells. The DN3 cells can be subdivided on the basis of the surface pre-TCR expression into expressors and non/low expressors. Referring back to work previously mentioned with regard to heterogeneity within the DN3 population it is possible that the non/low expressors of pre-TCR correspond to the pre-β-selection E cells and the higher expressors correlate with the post-β-selection L cells (Hoffman et al., 1996). DN3 cells are the first developmental stage at which the pre-TCR is detected on the surface. This fits with the above mentioned analysis of TCRβ chain rearrangements which are completed at this stage (Godfrey et al., 1993). pTa deficient mice have been generated and have a major block in thymocyte development (Fehling et al., 1995). Total thymocyte cellularity is reduced by 10-fold and development is partially blocked at the DN3 to DN4 transition. Virtually no DN4 cells are present and cells accumulate at the DN3 stage. DP and SP cells are present but in considerably lower numbers compared to wild type.
As previously mentioned the preTCR-CD3 complex is composed of the rearranged TCRβ chain, the pTα and associated CD3 subunits. The cytoplasmic domains of the CD3 subunits are larger than those of the TCRβ and pTα chains and are involved in coupling the TCRβ-pTα heterodimer to intracellular signalling machinery. The identity of the CD3 chains present in the pre-TCR remains controversial. The CD3-ε and γ polypeptides have been found associated with the pre-TCR complexes in pre-T cell lines but the CD3-δ and ζ have been more difficult to detect (Groettrup et al., 1992; Jacobs et al., 1994). The blockade in thymocyte development seen in RAG deficient mice can be overcome by injection with anti-CD3 antibodies (Levelt et al., 1993; Shinkai and Alt 1994). This implies that CD3 components are responsible for at least part of the signal transduction capacity of the pre-TCR. The importance of the various CD3 chains has been studied via the generation of mutant mice. A number of extensive reviews have been written recently describing these mice so this will be mentioned only briefly here (for review see Malissen et al., 1999). Two independently generated strains of CD3ε deficient mice have been analysed (Malissen et al., 1995; Wang et al., 1998b). These mice were blocked in thymocyte development at the DN 3 stage again at the same stage as RAG deficient mice. On further analysis the targeting strategies used to generate these mice were seen to affect the expression of the CD3 γ and δ genes also; these are therefore mutants of the entire CD3 γδε module. These mice have, however, formally demonstrated that some CD3 components are essential for the assembly or function of the pre-TCR. Reconstitution of CD3δ expression in the low percentage of thymocytes from these CD3ε deficient mice that retained CD3γ expression resulted in CD3ε-deficiency alone (Wang et al., 1999). These CD3ε deficient thymocytes are again severely, but not completely, blocked at the DN3 stage. Mice deficient for both CD3 γ and CD3 δ genes are also blocked in thymocyte development at the DN3 stage (Wang et al., 1998a). Therefore, the progression beyond the DN3 stage of thymocyte development depends on signals triggered by the pre-TCR-CD3 complex. Studies involving RAG deficient, TCRβ deficient, mice deficient for components of the DNA-end-joining reactions and SCID mice have shown the importance of successful rearrangement of the TCRβ gene in thymocyte development. Analysis of pTα
deficient mice has pointed to the essential role for this chain as a partner for the 
TCRβ chain, along with the CD3 components within the pre-TCR in this process. 
The signal transduction downstream of the pre-TCR is described in more detail 
in section 3.1.4.

1.4. The role of the αβ-TCR in thymocyte development

1.4.1. Positive and Negative selection

The role of the pre-TCR in the DN to DP transition has been discussed. 
The next checkpoint in T cell development, the differentiation of DP cells into 
mature SP cells, is mediated by the αβTCR-CD3 complex. DP cells have a life 
span of up to 4 days (Egerton et al., 1990) during this time DP cells undergo the 
processes of positive and negative selection based on the reactivity of the 
αβTCR-CD3 complexes with MHC molecules. Less than 2% of the cells that 
enter the thymus leave as mature SP cells. The majority of DP cells expressing 
an αβTCR cells die within the thymus due to their inability to recognise self-
MHC-peptide complexes on the thymic stroma. These cells die by a process 
termed death by neglect. Surh and Sprent were the first to demonstrate these 
cells die by apoptosis, showing apoptotic cells scattered throughout the cortex 
(Surh and Sprent 1994). The remaining cells undergo negative selection 
deletion) or positive selection and differentiate into single positive CD4 or CD8 
cells. The level of TCR signals, which is mainly affected by the avidity between 
the TCR and self-peptide/MHC and the contribution of co-receptors CD4 and 
CD8 determines the fate of thymocytes. Negative selection eliminates from the 
thymus DP cells with αβTCRs that react with high affinity to self-peptide/MHC 
within the thymus. Thymocytes with αβTCRs that recognise self MHC with low 
affinity are positively selected to survive and differentiate into TCR high CD4 or 
CD8 SP T cells (for review see Saito and Watanabe 1998). The signalling 
pathways for positive and negative selection are still being elucidated. The 
protein tyrosine kinases (PTKs) ZAP-70 and p56Lck are known to be important. 
The role for p56Lck in these processes was identified by the generation of 
transgenic mice that express a dominant negative p56Lck transgene under the 
control of the p56Lck distal promoter. Over-expression of the catalytically
inactive p56Lck led to inhibition of both positive and negative selection in these mice (Hashimoto et al., 1996). Mice deficient for ZAP-70 or for the protein tyrosine phosphatase CD45 also show the importance of these enzymes in positive and negative selection (Kishihara et al., 1993; Negishi et al., 1995; Byth et al., 1996). As we shall see later these enzymes are tightly associated (with CD45 regulating the activity of p56Lck and ZAP-70) and are vital in thymocyte development. A member of the nuclear hormone receptor superfamily of transcription factors, Nur77, has also been implicated in negative selection. Nur77 expression is upregulated in response to TCR engagement in immature thymocytes and in T cell hybridomas (Liu et al., 1994; Woronicz et al., 1994). These studies also showed that expression of a dominant negative or anti-sense form of Nur77 prevented apoptosis in TCR stimulated cells. Mice expressing a dominant negative form of Nur77 in thymocytes also have severe defects in TCR-mediated death during negative selection (Calnan et al., 1995). Surprisingly mice deficient for Nur77, however, display no defects in TCR-mediated death (Lee et al., 1995). This may be due to the presence of redundancy within the system.

1.4.2. CD4/CD8 lineage choice

As described above, during positive selection T cells down-regulate the expression of CD4 or CD8 and develop into CD4+ or CD8+ SP cells. DP thymocytes bearing MHC- class II specific αβTCRs will usually develop into CD4 SP cells while those with a MHC class I specific αβTCRs will usually develop into CD8 SP T cells so that the TCR nearly always matches the MHC specificity. Two main models have been proposed to explain how this choice between lineages is made (von Boehmer 1996). The first is termed the instructive model and proposes that the class of MHC expressed by the cell determines commitment. T cells with MHC class I restricted TCRs bind to peptide and the co-receptor CD8 is engaged, resulting in down-regulation of CD4. T cells with MHC class II restricted TCRs bind to peptide and the co-receptor CD4 is engaged and then CD8 is down regulated. Recently this model has been modified to include the idea that the signals that induce CD4 or CD8 cells choice differ in strength. The CD4 receptor is thought to deliver a strong signal to
induce the generation of CD4 cells while the signal delivered by the CD8 receptor is thought to be weaker (for review see Singer et al., 1999). The second model is the stochastic model, which is based on the down-regulation being a random event following TCR and co-receptor engagement, when expression of one or other co-receptor is terminated, regardless of the MHC specificity of the initiating signal. This model has since been adapted to the stochastic/selection model whereby TCR and co-receptor engagement results in the cell randomly down-regulating one co-receptor, after which the cell receives an additional rescue signal to differentiate into mature CD4 or CD8 T cells.

1.4.2.A. The Role of the PTK p56Lck in CD4/CD8 lineage choice

Various experiments have provided evidence for and against these proposals, and have led to numerous other models being suggested. The mechanisms behind CD4 or CD8 lineage choice however remain unresolved. The PTK p56Lck is involved in these processes. p56Lck associates with the coreceptors CD4 and CD8 and it is proposed that co-engagement of the TCR and co-receptor to an MHC molecule allows the associated p56Lck to interact with other signalling molecules associated with the TCR leading to downstream signalling (Veillette et al., 1988). p56Lck associates more efficiently with CD4 than CD8, with over 25% of CD4 and only 2% of CD8 surface molecules associated with p56Lck (Wiest et al., 1993). One theory is that the amount of p56Lck signal brought by the co-receptor, so determining the signal strength, could play a role in CD4/CD8 lineage choice. This has been verified by the study of transgenic mice with altered p56Lck activity (Hernandez-Hoyos et al., 2000). Transgenic thymocytes that expressed a class-II restricted TCR developed into CD8 T cells when p56Lck activity was reduced and thymocytes expressing a class-I restricted TCR developed into CD4 cells when p56Lck activity is increased. The level of the p56Lck signal therefore is important in lineage decisions. Work by Basson et al has further advanced these findings demonstrating that the maturation of CD4 cells occurs upon the recruitment of p56Lck to the TCR-CD3 complex, whereas CD8 maturation can be induced by CD3 ligation in the absence of p56Lck (Basson et al., 1998). Indirect evidence also supports this since maturation of CD4 SP
cells is reduced when p56Lck activity was compromised, namely in CD45-deficient thymocytes (Basson et al., 1998). Recent work utilising inducible-p56Lck-expressing transgenic mice has confirmed a central role for p56Lck in positive selection to the CD4 lineage but a less important role in positive selection to the CD8 lineage (Legname et al., 2000).

1.4.2.B. The Role of Notch in CD4/CD8 lineage choice

Signals from receptors other than those recognising MHC molecules have also been proposed to influence CD4/CD8 commitment, for example the Notch family of receptors have been implicated in this lineage choice. Notch signalling is involved multiple steps in T cell development, including a previously discussed role in T cell commitment (Radtke et al., 1999). Notch1 expression is developmentally regulated within the thymus, being expressed at the highest level in DN thymocytes, the lowest level in DP cells and at an intermediate level in SP cells (Hasserjian et al., 1996). What evidence is there for a role for Notch signalling in CD4/CD8 lineage choice? Transgenic mice expressing an activated form of Notch (Notch IC) develop more CD8 cells than CD4 cells suggesting, that Notch signalling may promote CD8 lineage choice (Robey et al., 1996). Expression of the NotchIC transgene in MHC class-1-deficient mice demonstrated that Notch activity permits the development of CD8 cells even in the absence of MHC class 1. However Notch signalling was not sufficient to allow CD8 cells to develop in the absence of both MHC class I and class II molecules. These results suggest that MHC ligation is required for a developing thymocyte to receive the Notch signal. A model was proposed as follows; DPs that are MHC class I restricted receive a Notch signal and develop into CD8 SP T cells whereas DPs that are MHC class II restricted do not receive a Notch signal and develop into CD4 SP T cells. This theory is backed up by the discovery that Hes-1, a target of Notch signalling, binds to a functional site in the CD4 silencer and that overexpression of Hes-1 results in downregulation of CD4 expression (Kim and Siu 1998). This indicates that signalling through Notch may directly control expression of the CD4 gene, so providing a possible mechanism for the role of Notch in CD4/CD8 lineage choice.
However, further work has suggested that Notch is involved in the generation of both CD8 and CD4 SP cells (Deftos et al., 1998). Activation of Notch-1 signalling was shown to lead to resistance to glucocorticoid-induced apoptosis and upregulation of other markers that correlate with DP maturation, including increased expression of Deltex (Deftos et al., 1998). Deltex has previously been shown to act as a positive regulator of Notch signalling (Matsuno et al., 1995). Since CD4 and CD8 SP T cells were shown to express high levels of Deltex Deftos and colleagues suggested that Notch signalling is involved in the maturation of CD4 as well as CD8 SP T cells. Recent studies indicate a role for Notch 1 later in these processes, after lineage fate is decided, in CD8 but not CD4 lineage progression (Yasutomo et al., 2000). This work also points to the importance of the duration of the co-receptor-induced T cell receptor dependent signalling. Therefore, the contribution of the signals from the TCR, the length and strength of these signals, as well as the role of signals from other co-receptors and the involvement of general cell fate regulators such as Notch in CD4/CD8 lineage choices is as yet unknown (for review see Singer et al., 1999).

1.5. Apoptosis and the expression of survival factors within the thymus

Apoptosis plays a crucial role in the maintenance of an efficient immune system. There are two main points during development when thymocytes are susceptible to apoptosis. Cells that successfully rearrange and express a pre-TCR composed of the TCRβ chain, the pTα and CD3 components progress from the DN3 to DN4 stage. Cells that fail to express a functional pre-TCR are thought to die by apoptosis apparently due to a lack of signalling from the pre-TCR (Penit et al., 1995) and the presence of death receptor signalling (Newton et al., 2000). DN4 cells that survive the pre-TCR checkpoint proliferate and differentiate into DP cells. Following rearrangement of the TCRα chain genes and expression of an αβTCR these DP cells then undergo positive and negative selection or death by neglect based on the reactivity of the αβTCR-CD3 complexes with MHC molecules. The majority of DP thymocytes die within the thymus due to failing positive selection or due to being negatively selected, so allowing for the
removal of unnecessary or potentially dangerous cells. The symptoms of apoptosis are viability loss accompanied by other features including cytoplasmic blebbing, chromatin condensation, relocation of phosphatidylserine residues from the inner to the outer aspect of the plasma membrane and DNA fragmentation (for a review see Cory 1995). Immature DPs are extremely sensitive to glucocorticoid (Cohen and Duke 1984), \( \gamma \) -radiation (Sellins and Cohen 1987) and to anti-CD3/TCR (Smith et al., 1989) induced apoptotic cell death. Multiple pathways exist that lead to a thymocyte undergoing apoptosis, a few of the genes involved will now be mentioned.

### 1.5.1. Bcl-2 family proteins

One of the first regulators of apoptosis to be identified was Bcl-2, which is now known to be a member of a growing family of proteins. This family can be divided into promoters of apoptosis, such as Bad, Bax and Bik, and inhibitors of apoptosis, such as Bcl-2, Bclx\(_L\) and Mcl-1 (for reviews see Reed 1998). The Bcl-2 gene was first discovered at the chromosomal breakpoint t(14;18) in neoplastic B cells (Tsujimoto et al., 1984) and was later shown to block programmed cell death in selected haematopoietic cell lines following cytokine deprivation (Vaux et al., 1988). Bcl-2 is expressed at high levels in DN thymocytes, low levels in DP cells with its expression being upregulated in SP cells (Veis et al., 1993; Moore et al., 1994). Overexpression of Bcl-2 within the thymus results in protection of immature thymocytes from a variety of death stimuli including DNA damage and glucocorticoid-induced apoptosis (Sentman et al., 1991; Strasser et al., 1991; Siegel et al., 1992). Thymocytes from these Bcl-2 transgenic mice remain sensitive to Fas (CD95)/Fas-L induced cell death, indicating that these are distinct pathways of apoptosis (Strasser et al., 1995). In contrast to the expression of Bcl-2, Bclx\(_L\) is expressed at a maximum level in DP cells (Grillot et al., 1995; Ma et al., 1995). Again thymocytes overexpressing Bclx\(_L\) are resistant to apoptosis induced by a number of signals, including DNA damage and glucocorticoid treatment. Interestingly Bclx\(_L\) -overexpressing thymocytes are also protected from Fas/Fas-L induced cell death, in contrast with Bcl-2-overexpressing thymocytes (Zhang et al., 1996). The distinct expression patterns of these two proteins implies they function as survival factors during different
phases of thymocyte development to resist different death inducing signals. It appears that DN thymocytes downregulate Bcl-2 expression and induce Bclx_L expression upon differentiating into DP cells (Veis et al., 1993; Moore et al., 1994; Grillot et al., 1995; Ma et al., 1995). These DP cells lack Bcl-2 expression and are vulnerable to apoptosis; their survival may depend on the maintenance of Bclx_L expression.

Other members of the family such as Bad, were discovered by virtue of their interaction with Bcl-2. Bad and Bax are thought to act pro-apoptotically via binding Bclx_L and Bcl-2, resulting in an antagonistic effect on their death repressing activity. Overexpression of Bad in an IL-3 dependent cell line revealed its pro-apoptotic affect (Yang et al., 1995). The expression of Bad is low in thymocytes, increasing greatly after apoptosis (Mok et al., 1999). Mice expressing a Bad transgene under the CD2 promoter have been generated (Mok et al., 1999). These mice have a severely reduced number of T cells and those that are present are highly sensitive to apoptotic stimuli, including γ-radiation, Dexamethasone treatment and additionally Fas/Fas-L induced cell death. Transgenic mice overexpressing Bax again develop a reduced number of mature T cells that are more sensitive to apoptosis (Brady et al., 1996). These cells are not, however, more susceptible to Fas/Fas-L induced apoptosis, in contrast to thymocytes from the Bad transgenic mice. Thus, a link between the Fas/Fas-L mediated pathway and both Bad and Bclx_L signalling exists. Bad has been shown to bind preferentially to Bclx_L rather than Bcl-2. This binding removes the protective function of Bclx_L and so accelerates Fas/Fas-L induced apoptosis (Yang et al., 1995).

1.5.2. Roles for Nur-77 and Notch signalling in apoptosis

A role for the nuclear hormone receptor, Nur-77, and for Notch signalling in the control of thymocyte cell death has already been mentioned. To reiterate, the expression of Nur-77 and its related transcription factor Nor-1 is upregulated during apoptosis induced by TCR-engagement (Liu et al., 1994). Expression of either a dominant negative or an antisense Nur-77 construct resulted in reduced TCR-mediated cell death (Liu et al., 1994; Woronicz et al.,
Transgenic mice that constitutively express wild-type Nur-77 within the thymus show a significant reduction in the number of thymocytes and peripheral T cells and accumulate apoptotic cells (Calnan et al., 1995; Weih et al., 1996). The second of these studies revealed an increased level of \( \text{Fas-L} \) mRNA and protein in these Nur-77 overexpressing thymocytes, indicating that one apoptotic pathway triggered by Nur-77 expression is the Fas/Fas-L signalling pathway (described below). However, analysis of thymocyte death in Nur-77 deficient mice revealed no differences between the extent or the rate of thymocyte death in comparison to cells from wild-type mice, indicating that Nur-77 is not required for TCR-mediated apoptosis (Lee et al., 1995).

Experiments employing retroviral transfection of constitutively active NotchIC constructs have shown that Notch signalling inhibits glucocorticoid-induced cell death in a T cell hybridoma (2B4.11) and a thymic lymphoma cell line (AKR1010) (Deftos et al., 1998). Bcl-2 expression was upregulated in AKR1010 but not 2B44.11 cells and both cell types showed increased levels of Deltex. In addition, thymocytes from transgenic mice expressing a truncated, constitutively active Notch-1 expressed under the p56\(^{\text{ck}}\) proximal promoter showed increased resistance to glucocorticoid-induced apoptosis (Deftos et al., 1998). This suggests that Notch signalling plays a role in protecting developing thymocytes from glucocorticoid-induced apoptosis. Work by Jehn et al has also shown that constitutively active forms of Notch-1 inhibit apoptosis (Jehn et al., 1999). In this case activated Notch was shown to inhibit Nur-77-dependent apoptosis. Expression of activated Notch-1 resulted in repression of Nur-77 transcription. Combined with the fact that Notch had been identified as a Nur-77 binding protein in a yeast two hybrid screen this suggests a possible mechanism for the antiapoptotic effect of Notch signalling (Jehn et al., 1999). Notch signalling may therefore regulate apoptosis during thymocyte maturation by directly interacting with members of the nuclear receptor superfamily and so have an effect on downstream gene targets. Whether Notch-1 physically interacts with the glucocorticoid receptor in this way is as yet unknown. (For a full review on the role of Notch in apoptosis see Miele and Osborne 1999).
1.5.3. Other genes involved in regulation of apoptosis within the thymus

As already indicated other pathways regulating apoptosis within developing thymocytes exist. These include the previously mentioned Fas/Fas-L mediated pathway, which will be mentioned briefly here. The Fas receptor belongs to the tumour necrosis factor (TNF) receptor family. Included in this rapidly growing family are TNF-R1, DR3 (APO-3/TRAMP), DR4 (TRAIL-R1) and DR5 (TRAIL-R2). Fas/Fas-L induced cell death has been shown to be important for peripheral deletion of activated mature T cells at the end of an immune response. In addition it plays a role in killing of virus-infected cells and cancer cells by cytotoxic T cells and NK cells. Fas/Fas-L cell death is also important for the removal of inflammatory cells at "immune privileged" places such as the eye (for review see Nagata and Golstein 1995; Ashkenazi and Dixit 1998). The importance of Fas/Fas-L cell death within the thymus is unclear. Fas-L is predominantly expressed on activated T cells but is also detected within the thymus (Suda et al., 1995). The receptor Fas is more widely expressed in various tissues such as the thymus, kidney, heart and ovaries. Fas is expressed at a low level on DN thymocytes but is highly expressed on DP and SP thymocytes (Ogasawara et al., 1995). Fas-L binds to Fas and induces apoptosis of Fas bearing cells. DP thymocytes have been seen to selectively undergo apoptosis upon treatment with an anti-Fas antibody (Ogasawara et al., 1995). The importance of this system is clearly seen in the naturally occurring Fas receptor mutant lpr mice and Fas-L gld mice (for review see Nagata and Suda 1995). These mice accumulate CD4-CD8-CD3+ T cells and suffer from autoimmunity and lymphadenopathy. Negative selection was originally described as normal in the lpr mice but more recent reports indicate that both positive and negative selection are Fas-dependent (Kishimoto et al., 1998; Kurasawa et al., 1999). Therefore, despite the high level of Fas expression in DP thymocytes and the fact that DP thymocytes have been seen to selectively undergo apoptosis upon treatment with an anti-Fas antibody (Ogasawara et al., 1995,) the role of Fas in negative selection is unclear.

The tumour suppressor p53 has also been shown to be required for certain forms of thymocyte cell death. Thymocytes from p53-deficient mice showed normal death responses to glucocorticoid and signals mimicking TCR
stimulation but were unable to die in response to γ-radiation (Lowe et al., 1993). Thus, p53 is required to couple signals received from γ-radiation to apoptosis effects downstream. The various pathways leading to thymocyte apoptosis and their importance in the maintenance of homeostasis of the immune system have been briefly mentioned.

1.6. Signal transduction via the αβTCR

As previously mentioned, the αβTCR-CD3 is composed of the polymorphic α and β subunits noncovalently associated with CD3ε, δ and γ and a dimer of ζ (referred to as CD3ζ) subunits. The TCR α and β chains are composed of V (variable) and C (constant) domains and are associated by disulphide bridges. The α and β chains are responsible for antigen binding and have only short cytoplasmic domains of 5 amino acids so are unlikely to be able to participate in intracellular signalling. The CD3 subunits have larger cytoplasmic domains and are responsible for intracellular signalling. The capacity of these units to participate in signal transduction is mediated by the cytoplasmic domains of the CD3 chains, each containing a motif termed an immunoglobulin receptor tyrosine-based activation motif (ITAM). The ζ chain also contains 3 of these motifs (for review see Weiss and Littman 1994). TCR recognition of antigen in the context of MHC molecules is believed to juxtapose the protein tyrosine kinase (PTK) p56Lck with the ITAMs of the CD3 subunits, leading to their efficient phosphorylation (DeFranco 1995). This tyrosine phosphorylation of the ITAMs induces binding of the tandem SH2 domains of ZAP-70 and recruitment of ZAP-70 into the TCR complex (Iwashima et al., 1994). This leads to phosphorylation and activation of ZAP-70 and consequently to phosphorylation of downstream targets (see Figure 1.4).

One protein that undergoes tyrosine phosphorylation following TCR activation is phospholipase C (PLCγ1). Tyrosine phosphorylation of PLCγ1 leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). The production of IP3 leads to the mobilisation of intracellular calcium stores and the activation of calcium binding proteins such as the serine/threonine phosphatase calcineurin. Calcineurin acts
Figure 1.4: A model for ITAM-mediated signal transduction showing putative roles for p56\(^{Lck}\), p59\(^{Fyn}\) and ZAP-70.
upon the transcription factor NFAT (nuclear factor of activated T cells) to allow it to translocate to the nucleus and regulate the expression of numerous downstream genes. The presence of DAG activates the serine/threonine protein kinase C (PKC) family. PTK activity results in the initiation of additional cascades of kinase activity including the activation of the Ras/MAPK (mitogen activated protein kinase) pathway. Other downstream pathways activated include those leading to IL-2 secretion and cytolytic activity (for review see Weiss and Littman 1994; Cantrell 1996).

1.6.1. PTKs implicated in TCR signalling

PTKs were first implicated in TCR signalling following the observation that treatment of T cells with Herbimycin A (which directly inhibits kinase activity and leads to degradation of Src PTKs) results in defective signal transduction following T cell activation (June et al., 1990). Herbimycin A treated T cells showed impaired TCR-mediated activation, tyrosine substrate phosphorylation and reduced PLCγ1 activity. A number of PTKs are implicated in TCR signalling, of these the role of p56Lck, p59Fyn and ZAP-70 will be discussed here. The potential role for p56Lck in T cell activation was first indicated by the demonstration that murine p56Lck is associated with the co-receptors CD4 and CD8 following antibody-mediated cell surface cross-linking and co-immunoprecipitates with CD4 and CD8 (Veillette et al., 1988; Barber et al., 1989). The engagement of the TCR as well as CD4 or CD8 with the same peptide antigen-MHC complex is often required to initiate a full response to antigen (Swain 1983). CD4/CD8:p56Lck complexes have been shown to directly phosphorylate components of the CD3 complex (Barber et al., 1989). Stable association of p56Lck with CD4 or CD8 is dependent on cysteine residues within the unique N-terminal region of p56Lck and complementary cysteine-containing sequences within the cytoplasmic domains of CD4 and CD8 (Turner et al., 1990). This direct association suggests an important role for p56Lck in TCR signal transduction.

This pathway was further elucidated by studies of a mutant Jurkat T cell line that failed to respond to stimulation through the TCR. These Jurkat cell
lines expressed normal levels of TCR and p59^{Fyn} and yet were unable to respond to anti-CD3 antibody treatment (which mimics TCR stimulation) (Straus and Weiss 1992). In one mutant line, JCaM1, this defect has been shown to be due to lack of endogenous p56^{Lck} activity. Expression of a wild type p56^{Lck} cDNA in JCaM1 restored the ability of the cell to respond to TCR stimulation. In addition, over-expression of a catalytically activated form of p56^{Lck} has been shown to enhance T cell responsiveness (Abraham et al., 1991c). CD4-negative MHC class II restricted mouse T cell hybridoma cells were infected with retroviruses expressing constitutively active F505 p56^{Lck}. Cells expressing this mutant showed an increase in tyrosine phosphorylation following anti-CD3 antibody treatment and an enhanced antigen-induced IL-2 response. Studies using CD4 co-receptors that are unable to bind to p56^{Lck} also argue for the importance of p56^{Lck} in lymphocyte signal transduction (Glaichenhaus et al., 1991). Of the CD4-deficient T cell hybridomas studied, only those transfected with forms of CD4 that maintained the ability to interact with p56^{Lck} were able to respond to antigen. Antibody mediated crosslinking of CD4 (to mimic interaction with its ligand, MHC II molecules) substantially elevates the kinase activity of p56^{Lck} and is associated with changes in tyrosine protein phosphorylation (Veillette et al., 1989a; Luo and Sefton 1990). Additional studies have demonstrated that the levels of p56^{Lck} mRNA and protein decline rapidly after T cell activation (Marth et al., 1987). This downregulation is closely correlated with the induction of cytokine production and is not directly linked to proliferation. In addition, T cell activation has been shown to result in modification of p56^{Lck} (Marth et al., 1989). These results suggest that p56^{Lck} functions in the control of early events in T cell activation, but it is not be the sole PTK activated by the TCR (for review see Chan et al., 1994). Biochemical and genetic evidence (see below) also suggest a role for p59^{Fyn} in the TCR signalling cascade. p59^{Fyn} activity increases upon TCR stimulation and, using sensitive kinase assays and mild detergents for solubilisation, p59^{Fyn} has been seen to co-immunoprecipitate with the TCR-ζ subunit (Sarosi et al., 1992; Tsygankov et al., 1992). The Syk family member ZAP-70 also associates with the TCR but unlike the previously mentioned Src
PTKs this requires TCR stimulation. ZAP-70 is rapidly recruited, via its SH2 domain, to the ζ and CD3ε subunits upon TCR stimulation (Chan et al., 1992).

1.6.2. p56Lck, p59Fyn and ZAP-70 deficient mice

The analysis of mice deficient for these PTKs has underpinned their role in thymocyte development and TCR signalling. p56Lck deficient mice exhibit a severe reduction in thymocyte cellularity with an incomplete arrest in thymocyte development at the DN3 stage (Molina et al., 1992). Thymi from these mice have ten to twenty times fewer DP thymocytes in comparison to wild type mice. The mature SP T cells that do develop show a reduced proliferative response to both CD3 and αβ TCR crosslinking in comparison to wild-type controls (Molina et al., 1992). Thymocytes from these mice show very little basal phosphorylation of the TCR ζ chain (van Oers et al., 1996a). In addition the level of TCR-inducible ζ, CD3-δ or ZAP-70 phosphorylation was severely reduced, although not completely abrogated. Analysis of γδ T cell development in the p56Lck deficient mice has revealed that p56Lck is also required for the thymic development of γδ T cells (Penninger et al., 1993; Kawai et al., 1995). Analysis of αβ T cell development is even more profoundly arrested in transgenic mice expressing a dominant negative p56Lck transgene, as evidenced by the complete lack of DP cells and a total developmental arrest at the DN3 thymocyte stage (Levin et al., 1993).

The partial block in thymocyte development in the p56Lck deficient mice and reduction but not abolition of ITAM phosphorylation suggests that p56Lck is important in the initiation of TCR signalling but that there may be some redundancy within the system. This implies that in the absence of p56Lck another kinase may cover its function. The obvious candidate for this role is p59Fyn. p59Fyn deficient mice exhibit normal thymocyte development (Appleby et al., 1992; Stein et al., 1992). However the SP thymocytes that develop in the p59Fyn deficient mice show poor proliferative responses and low mobilisation of intracellular calcium following anti-CD3 antibody treatment. Mature splenic T cells from these mice retained normal proliferative responses but show reduced
intracellular calcium mobilisation and IL-2 production. In contrast to p56\textsuperscript{Lck} deficient mice the level of basal phosphorylation of ζ and the TCR-induced phosphorylation of CD3-δ remains unchanged in thymocytes from p59\textsuperscript{Fyn} deficient mice (van Oers et al., 1996a). These data suggest that p56\textsuperscript{Lck} and p59\textsuperscript{Fyn} have overlapping roles in thymocyte development and in TCR signalling. In accordance with this mice deficient in both p56\textsuperscript{Lck} and p59\textsuperscript{Fyn} are completely arrested in thymocyte development at the DN pre-TCR-mediated stage (van Oers et al., 1996b). In addition, the expression of an activated p59\textsuperscript{Fyn} transgene in p56\textsuperscript{Lck} deficient mice restores the production of DP thymocytes and leads to an increased percentage of SP thymocytes (Groves et al., 1996).

ZAP-70 deficient mice are also severely arrested in thymocyte development. These mice have normal numbers of DP thymocytes but no SP thymocytes develop due to an inability of the DP cells to undergo positive or negative selection (Negishi et al., 1995). Furthermore, DP cells from the ZAP-70 deficient mice failed to apoptose upon TCR stimulation. The important role for ZAP-70 in TCR mediated signalling was first realised through studies of SCID patients with ZAP-70 mutations (Chan et al., 1994; Elder et al., 1994). These patients developed CD4 SP cells but no CD8 SP cells. The CD4 SP T cells were unable to synthesise IL-2 or to proliferate following TCR stimulation. A SCID patient has since been identified with a specific deficiency in p56\textsuperscript{Lck} expression although p59\textsuperscript{Fyn} and ZAP-70 are expressed at normal levels (Goldman et al., 1998). The involvement of these three PTKs in TCR-signalling is therefore established. For further discussion relating to the role of p56\textsuperscript{Lck} in allelic exclusion and in signalling downstream of the pre-TCR-CD3 complex see section 3.1.4.

Members of another non-receptor PTK family, the Tec family, have recently been implicated in TCR-signalling (for review see Schaeffer and Schwartzberg 2000). The Tec kinases are structurally similar to the Src kinases and members include Tec, Btk, Itk, Bmx, Rlk and Dsrc29. These kinases have previously been studied in connection with signalling downstream of antigen
receptors but their role in T cells was less well understood. Mice deficient for \textit{Itk} or \textit{Rlk} and \textit{Itk} have defective TCR responses including proliferation, cytokine responses and adaptive immune responses to \textit{Toxoplasma gondii} (Liu et al., 1998; Schaeffer et al., 1999). T cells from these mice have normal patterns of tyrosine phosphorylation upon TCR stimulation but show decreased calcium mobilisation and IP$_3$ production and a modest reduction in PLC$\gamma_1$ tyrosine phosphorylation. Therefore, in addition to the well documented roles for p$56^{Lck}$, p$59^Fyn$ and ZAP-70 members of the Tec family of PTKs are also implicated in TCR signalling.

\subsection*{1.6.3. The role of Protein tyrosine phosphatases in TCR signalling}

As described above TCR mediated signalling and numerous other signalling pathways in lymphocytes are co-ordinated by the action of PTKs. The activity of these PTKs is regulated by the protein tyrosine phosphatases (PTPases). The well characterised PTPases CD45 and SHP-1 (src homology 2 containing intracellular protein tyrosine phosphatase 1) will be mentioned here. The tyrosine phosphatase CD45 has been identified as a critical regulator of TCR signalling. CD45 is a transmembrane PTPase that comprises two intracellular PTPase domains and is expressed on almost all haematopoietic cells (for review see Neel 1997). The numerous studies performed have, however, produced conflicting data on the role of CD45 as either an activator or an inhibitor of this process. The primary function of CD45 is as a positive regulator of antigen receptor signalling via activating p$56^{Lck}$ and p$59^Fyn$ through the dephosphorylation of the negative inhibitory residues of these kinases. The main sites for phosphorylation are the tyrosine residues at amino acid 505 of p$56^{Lck}$ and 531 of p$59^Fyn$. Mutation of these tyrosine residues results in constitutive kinase activity (Reynolds et al., 1987). Phosphorylation of this residue by the tyrosine kinase p$50^{csk}$ (c terminal src kinase) inhibits the catalytic activity and desphosphorylation by CD45 stimulates kinase activity (Mustelin et al., 1989; Ostergaard and Trowbridge 1990; Okada et al., 1991). The importance of CD45 activity in TCR signalling is clear from the analysis of \textit{CD45} deficient T cell clones (for review see Neel 1997). These results have been confirmed by the generation of two lines of \textit{CD45} deficient mice. Kishihara \textit{et al.} targeted \textit{CD45}
exon six, resulting in all B cells and most T cells lacking CD45 expression (Kishihara et al., 1993). These mice are therefore not true nulls. Byth et al. targeted the isoform-common exon nine, producing true CD45-null mice (Byth et al., 1996). The two lines of CD45 deficient mice have similar phenotypes. These mice are immunocompromised, failing to generate effective cytotoxic T cell responses. They also have a four to ten-fold reduction in thymocytes and peripheral T cells. CD45 deficient DP thymocytes are severely impaired in their apoptotic response to TCR crosslinking and display reduced TCR-induced proliferative responses. Analysis of γδ T cell development in the CD45 deficient has revealed that CD45 is also required for the thymic development of γδ T cells (Kawai et al., 1995). In addition, a SCID patient has since been identified with a specific deficiency in CD45 (Kung et al., 2000). Therefore, the PTPase CD45 has a vital role in thymocyte development and in TCR signalling.

Another PTPase involved in the regulation of TCR signalling is SHP-1. Two naturally occurring SHP-1 mutations have been identified in mice; the moth-eaten mouse which is effectively SHP-1 deficient and the moth-eaten viable mouse which expresses two SHP-1 proteins with reduced PTPase activity (for review see Neel 1997). Thymocyte development in these mice is normal but thymocytes and T cells hyper-proliferate in response to anti-TCR signals (Pani et al., 1996). How SHP-1 regulates TCR signalling is unclear and has been confused by conflicting reports. Plas et al have reported that SHP-1 binds to ZAP-70 and reduces its activity (Plas et al., 1996). However, the levels of constitutively or inducibly phosphorylated proteins are increased and p56Lck and p59Fyn are hyperactive in the moth-eaten mice (Lorenz et al., 1996; Pani et al., 1996). Thus, although the mechanism by which SHP-1 regulates TCR signalling is not fully understood, the importance of this and other PTPases is clear.

1.6.4. The role of Adapter molecules in TCR signalling

Tyrosine phosphorylation of components of the TCR complex provides binding sites for a number of proteins, termed adapters, which lack intrinsic enzymatic activity but are important for coupling the tyrosine phosphorylated TCR/CD3 complex with downstream signalling pathways. These adapters mediate protein-protein interactions through a variety of functional domains.
These include src-homology-2 (SH2), src-homology-3 (SH3) and phosphotyrosine binding (PTB) domains. The SH2 domains and the PTB domains can interact with tyrosine-phosphorylated proteins and the SH3 domains bind to proline-rich regions of proteins. Adapter proteins also contain regions that allow binding domains of other proteins to recognise them. There are many of these adapter proteins that are involved in co-ordinating the interactions amongst signal transduction effector proteins downstream of TCR activation. These include Grb-2, LAT, SOS, SLP-76 and SLAP-130 that enhance antigen-receptor initiated transduction events and Cbl and SAP that function as negative regulators of signal transduction events initiated by the TCR. These will not all be discussed here (Norian and Koretzky Immunol 2000 Feb). A few adapter proteins of particular interest with respect to their interaction with p56Lck will now be mentioned.

One of the first adapter proteins identified was the growth factor receptor binding protein 2 (Grb-2). This protein contains one SH2 domain flanked on either side by a SH3 domain which are involved in complex formation of this adapter with other key regulatory molecules, including son-of-sevenless (SOS) and SH2 domain leukocyte protein of 76kDa (SLP-76). SOS is a homologue of Drosophila Ras GDP/GTP exchange factor (Bonfini et al., 1992). SOS can bind to the SH3 domains of several molecules, including Grb2, via the proline rich region at its carboxyl terminus (Li et al., 1993; Rozakis-Adcock et al., 1993). On recruitment to the membrane the guanine nucleotide exchanger SOS can then activate Ras. Activated Ras has a central role in IL-2 gene activation by regulating the MAPK pathway (Cantrell 1996). In addition to its interaction with Grb2, SOS has been shown recently to bind to the activated form of p56Lck (Park et al., 1998). This p56Lck -SOS interaction may also be involved in the regulation of the Ras pathway.

SLP-76 was first identified through its association with Grb-2. This haematopoietic cell-specific adapter protein is composed of an amino terminal region that contains multiple tyrosine residues, a central proline rich region and a carboxyl SH2 domain (Jackman et al., 1995). Biochemical analysis has revealed that SLP-76 associates with Zap-70, Vav, Grb-2, SLP-76-associated phosphoprotein, 130 kDa (SLAP-130) and other proteins (for review see
Koretzky, 1997). Importantly for the following discussion, SLP-76 has also been shown to bind directly and selectively to p56Lck (Sanzenbacher et al., 1999). Studies using a SLP-76 deficient Jurkat cell line revealed insights into the role of SLP-76 in activated T cells (Yablonski et al., 1998). T cell stimulation resulted in diminished calcium mobilisation, a lack of phosphorylation of PLCγ1 and a reduced TCR-dependent activation of Ras. Overexpression of SLP-76 in Jurkat cells resulted in enhanced TCR-mediated induction of nuclear factor of activated T cells (NFAT) and IL-2 promoter activity (Motto et al., 1996). These results implicate SLP-76 as a critical player in a number of signal transduction pathways initiated upon T cell activation. A vital role for SLP-76 in thymocyte development has been demonstrated by the generation of SLP-76 deficient mice (Clements et al., 1998; Pivniouk et al., 1998). These mice are completely blocked in thymocyte development at the DN3 stage; no DP or SP thymocytes develop. This developmental block in thymocyte development is more severe than the phenotype seen in mice lacking any single Src or Syk kinase. This indicates that unlike the Src and Syk family of kinases there appears to be no redundancy at the level of SLP-76 function in pre-TCR dependent signalling. Further investigations revealed that SLP-76 DN thymocytes are able to rearrange their TCRβ chain genes and express the pre-TCR on their surface (Aifantis et al., 1999). In vivo treatment with anti-CD3 (Pivniouk et al., 1998) or the introduction of a TCRαβ transgene (Aifantis et al., 1999) failed to cause expansion of DN thymocytes or developmental progression to the DP stage so implying a deficiency in SLP-76 results in impaired signal transduction from the pre-TCR. A role for SLP-76 in allelic exclusion is also alluded to since the introduction of a functional rearranged TCRαβ transgene into SLP-76 deficient mice did not result in cessation of rearrangement of endogenous TCRβ genes (Aifantis et al., 1999). In view of the direct association of SLP-76 with the src kinase p56Lck these roles for SLP-76 as a vital adapter protein in signalling downstream of both the pre-TCR and the αβTCR are well founded.

In addition to SLP-76 and SOS other adapter proteins have recently been described as directly interacting with p56Lck. Firstly, an adapter protein that interacts with the SH2 domain of p56Lck, called LAD (Lck-associated adapter protein) will be mentioned (Choi et al., 1999). LAD contains several protein-
protein interaction domains including a zinc finger motif, an SH2 and an SH3 domain and several phosphotyrosine sites. This adapter protein was shown to associate with p56\textsuperscript{Lck} and to be phosphorylated upon TCR stimulation. Overexpression of a dominant negative LAD blocked the IL-2 promoter driven transcriptional activation following TCR stimulation. In addition, LAD was redistributed to the plasma membrane upon stimulation (Choi et al., 1999). These results indicate that LAD physically associates with p56\textsuperscript{Lck} and plays an important role in the p56\textsuperscript{Lck}-dependent T cell signalling. A second putative p56\textsuperscript{Lck} associated adapter protein is LckBP1/HS1, a p56\textsuperscript{Lck} binding protein expressed solely in haematopoietic cells. LckBP1/HS1 contains an SH3 domain, two proline rich regions and becomes tyrosine phosphorylated upon B cell and T cell antigen-receptor cross-linking (Yamanashi et al., 1993; Takemoto et al., 1995). Immunoprecipitation analyses have confirmed the association of LckBP1/HS1 with p56\textsuperscript{Lck} (Takemoto et al., 1995). LckBP1/HS1 deficient mice show impaired proliferative responses and a disturbance in normal repertoire selection (Taniuchi et al., 1995). LckBP1/HS1 looks likely to be an additional adapter protein involved in mediating intracellular signals from p56\textsuperscript{Lck}.

The above experiments illustrate the importance of PTKs, in particular the Src family of PTKs, associated adapter proteins and PTPases in thymocyte development and TCR signalling. This discussion leads on to the first aim of my thesis, that of investigating the expression pattern of the PTK p56\textsuperscript{Lck} throughout thymocyte development.

1.7. Transcription factors involved in lymphoid commitment

As previously discussed, it is thought that the co-ordinated regulation of the developmental potential of haematopoietic progenitor cells is determined by the repression of lineage-inappropriate genes and the activation of lineage-specific genes. This results in the suppression of alternative fates by selection of the gene expression program of a single lineage (Enver and Greaves 1998). The first evidence to indicate this hypothesis was the finding that single cells of a single lineage expressed both \textit{\textbeta}-\textit{globin} and the \textit{myeloperoxidase} genes before they
commit to either erythroid or granulocyte lineages (Hu et al., 1997). The role of Notch signalling in control of cell fate decisions during thymocyte development, through the regulation of expression of target genes, has been discussed. Notch signalling has been implicated in CD4 or CD8 lineage choice and in control of apoptosis (Robey et al., 1996; Deftos et al., 1998; Jehn et al., 1999). Roles for Notch signalling in the αβ/γδ developmental choice have also been elucidated where increased Notch activity favours the development of αβ rather than γδ T cells (Washburn et al., 1997). Conditional inactivation of the Notch gene in thymocytes has revealed a role for Notch 1 in the specification of T cell fate (Radtke et al., 1999).

Numerous transcription factors have been implicated in the direction of uncommitted cells to develop into specific cell types, presumably by regulating expression of genes whose products affect cell fate or function. With respect to the commitment to the lymphoid lineage PU.1, GATA-3 and Ikaros are involved. Recently the B-lymphoid specific transcription factor Pax-5 has been identified as playing an important role in B cell lineage commitment (Nutt et al., 1999). A transcription factor playing this definitive role in T cell commitment is yet to be identified; however, those implicated will now be discussed.

The Ikaros family of lymphoid restricted zinc-finger nuclear proteins was first identified by virtue of Ikaros binding to the CD3δ enhancer (Georgopoulos et al., 1992). Ikaros gene expression is detected first in the embryonic yolk sac, the site of primitive haematopoiesis at embryonic day 8 (E8) in mice and later in foetal liver and in the thymus. Ikaros RNA is expressed in HSC populations, B cell progenitors in BM and in mature T, B and NK cells (for review see Georgopoulos et al., 1997). This pattern of expression suggested that Ikaros might be a regulator of lymphoid development. This role was established when mice with a carboxyl-terminal deletion in the Ikaros gene were generated (Wang et al., 1996a). In these Ikaros-deficient mice B cell development was arrested before the immature pre-B cell precursor stage and NK cells were absent. Foetal thymi lacked any lymphoid cells but within the first week after birth mature T cells developed. Erythropoiesis and myeloid cell differentiation appeared to be normal. Mice expressing a dominant negative form of Ikaros, due
to an amino-terminal deletion, fail to generate any definitive T, B or NK cells; in contrast, development of erythroid and myeloid lineages was unaffected (Georgopoulos et al., 1994). These results indicate a role for Ikaros in control of commitment of cells into the lymphoid lineage. In addition, a role for Ikaros in thymocyte development has been alluded to through breeding of Ikaros-deficient mice with RAG-deficient mice (Winandy et al., 1999). Unexpectedly, thymocytes in these double mutant mice can differentiate from the DN to the DP stage despite lack of expression of the pre-TCR. However the proliferation usually seen following this differentiation is absent, resulting in a severely hypocellular thymus. The Ikaros deficient mice have also been crossed with TCRα deficient mice, which are usually blocked in thymocyte development at the DP stage. In these double mutant mice cells that have downregulated expression of CD4 or CD8 are present that appear to represent intermediates between DP and SP thymocytes (Winandy et al., 1999). These results led the authors to theorise that the Ikaros proteins set thresholds at the pre-TCR and αβTCR mediated checkpoints in T cell differentiation. Without Ikaros activity these thresholds appear to be reduced, allowing thymocytes to differentiate from DN to DP and from DP to SP low cells without the appropriate pre-TCR or αβTCR signalling respectively. Ikaros proteins are therefore critically involved in various stages of lymphopoiesis, from T cell commitment, to control of proliferation, to control of differentiation through checkpoints in thymocyte development.

GATA transcription factors are defined by a conserved DNA-binding domain, which recognises a GATA-consensus motif. Three of these family members have emerged as possible regulators of haematopoietic gene expression, GATA-1, 2 and 3. Of these GATA-3 has been implicated in commitment to the T cell lineage. Expression of GATA-3 in the haematopoietic lineages is restricted to T and NK cells (Ho et al., 1991). GATA-3 was first identified due to binding to the TCRα gene enhancer and has been implicated in the T cell specific regulation of several cell surface molecules, including the TCRα, β and δ (Kuo and Leiden 1999). GATA-3 deficient mice show embryonic lethality at E12 displaying anaemia, abdominal haemorrhages and brain and spinal cord abnormalities (Pandolfi et al., 1995). The early embryonic lethality
has precluded an analysis of the role of GATA-3 in thymocyte development in these mice. ES cells containing a homozygous GATA-3 mutation were analysed using RAG deficient blastocyst complementation to see if GATA-3 deficient thymocytes can develop when in the environment of normal thymic stroma (Chen et al., 1993). The resulting chimeric mice developed mature erythroid, myeloid and B cell lineages but failed to generate thymocytes or mature T cells (Ting et al., 1996). This demonstrates that GATA-3 is required for the development and or survival of the earliest committed DN thymocytes or their precursors. In addition to its role in commitment to the T cell lineage GATA-3 also has a role in Th1/Th2 developmental choice. GATA-3 is specifically upregulated in Th2 cells and has been shown to activate expression of Th2 cytokines IL-4 and IL-5 (Zheng and Flavell 1997). Recent work has demonstrated that GATA-3 can induce the expression of these Th2 cytokines even in committed Th1 cells and redirect them into Th2 cells (Lee et al., 2000).

The third gene product that is required for development of early lymphoid progenitors is PU.1, a member of the ETS gene family of winged helix-loop-helix transcription factors. The ETS family of transcription factors are identified by the presence of an 85 amino acid region of homology that mediates DNA binding to a purine-rich core motif (GGAA/T). PU.1 is specifically expressed in monocytic and B and T cell lineages (for review see Bassuk and Leiden 1997). Disruption of the PU.1 gene has resulted in two different phenotypes depending on the targeting strategy used. PU.1 deficiency, in the first mice analysed, resulted in late embryonic lethality of anaemic embryos at E18 (Scott et al., 1994). Histological analysis of the foetal liver and thymus revealed a lack of lymphoid and myeloid precursors but the presence of cells of erythroid lineages. The generation of chimeric mice using ES cells from these mice confirmed these results (Scott et al., 1997). PU.1 deficient ES cells failed to contribute to myeloid or lymphoid lineages but showed a low level of participation in foetal erythropoiesis. In the adult chimeras PU-1 was seen to be essential for the generation of all haematopoietic lineages. The second line of PU.1 deficient mice showed a less severe phenotype (McKercher et al., 1996). Upon treatment with antibiotics these mice survived to 2 weeks of age and, although they lacked macrophages and mature B cells, erythropoiesis was
normal and thymocytes were found. Thymocyte development was delayed but did occur in these mice, resulting in a five to ten-fold reduction in overall thymocyte number. A recent study concentrating on thymocyte development in the first line of PU.1 deficient mice also indicated that PU.1 may not be vital for thymocyte development (Spain et al., 1999). Studies of embryonic mice revealed the presence of T cell progenitors, blocked in differentiation prior to commitment, in PU-1 deficient embryos. In addition, when placed into FTOC a fraction of PU-1 deficient thymi developed beyond the point of commitment and in certain cases to maturity. Therefore, although PU-1 is not essential for thymocyte development it plays a role in efficient commitment and possibly in early differentiation of T cell progenitors.

1.8. Transcription factors involved in thymocyte development

Haematopoiesis is controlled by signals that activate lineage specific genes, allowing for cellular proliferation, survival and differentiation. In addition to the above mentioned PTKs and signalling molecules, other genes encoding for T cell specific transcription factors are important for T cell development, some of which will now be introduced. The identification of transcription factors required in T cell development has been limited by the fact that a number of the genes that would be obvious candidates are actually essential early on in haematopoiesis by the HSCs. Thus deficiencies result in a complete block in haematopoiesis and embryonic lethality so precluding the study of the role of these factors in T cells. This has proved a serious limitation.

C-myb was originally cloned as a cellular homologue of the retroviral oncogene v-myb. It is expressed in progenitors of erythroid, myeloid and lymphoid lineages at a high level; this level decreases when the cells differentiate (for review see Tenen et al., 1997). Embryos deficient for c-myb die at days E13-E15 and show reduced liver erythropoiesis, although embryonic erythropoiesis in the yolk sac was intact (Mucenski et al., 1991). These data are consistent with the idea of a role for c-myb in maintaining the proliferative state of haematopoietic progenitor cells. Since these mice die before definitive
haematopoiesis studying the role of c-myb in thymocyte development with these c-myb deficient mice has not been possible. However, the generation of chimeric mice using ES cells from these mice has elucidated a role for c-myb in the control of early thymocyte development (Allen et al., 1999). These RAG/c-myb chimeras fail to develop B cell or macrophage/monocyte precursors and the thymocyte precursors that develop are arrested in development at the DN1 stage prior to TCR rearrangement. Therefore, c-myb is important at multiple stages of haematopoiesis and is required at an early stage of thymocyte development.

Previous discussion has covered the role of one ETS family member, PU.1, in myeloid and lymphoid precursor development. Other members of the ETS family are also involved in T cell development and function. Ets-1 is a 56kDa protein that binds to sites in the TCRα and β enhancers and has been shown to bind co-operatively with the AP-1 transcriptional complex to activate cellular growth factor responses (for review Bassuk and Leiden 1997) Ets-1 is expressed at high levels in B, T and NK cells. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Ets-1 expression within thymocyte subsets revealed an absence of expression in the early uncommitted DN1 population but an clear expression in DN2 cells that are committed to the T cell lineage (Spain et al., 1999). Ets-1 expression was highest in DP and SP thymocytes and in mature peripheral T cells. Ets-1 deficient ES cells were used to generate RAG-2 chimeric mice and revealed that, although Ets-1 is not required for the development of mature T or B cells, other defects are seen (Bories et al., 1995; Muthusamy et al., 1995). The T cells in these mice were present in reduced numbers, displayed a defect in TCR-mediated activation and are highly susceptible to cell death in vitro. B cells were present in normal numbers but a higher percentage of them had differentiated into IgM plasma cells, resulting in a five to ten fold elevated serum IgM levels. Significantly reduced numbers of Natural Killer (NK) cells were seen in these mice and splenocytes lacked detectable cytolytic activity against NK cell targets in vitro (Barton et al., 1998). Thus, Ets-1 is required for the development of NK cells and implicated in the maintenance of the normal pool of T and B cells.
T cell factor 1 (Tcf-1) and lymphoid enhancer-binding factor 1 (Lef-1) are members of the HMG (high-mobility group) protein family. These transcription factors were all isolated by their ability to bind to the enhancer elements of CD3-ε (for review see Kuo and Leiden 1999). Tcf-1 and Lef-1 may both function by bending the DNA elements to which they bind so allowing the assembly of multiple transcription factors on enhancers such as the TCRα enhancer (Love et al., 1995). Tcf-1 and Lef-1 are expressed in non-haematopoietic and haematopoietic cells, including expression throughout T cell development. Tcf-1 mRNA is expressed slightly earlier than Lef-1 being found from DN1 cells and reaching a maximum in the immature single positive (ISP) cells (Verbeek et al., 1995). Lef-1 mRNA is found from the DN3 subset onwards and is still present at the DP stage. Mice lacking a functional Tcf-1 gene develop normally but they have a partial block in thymocyte development (Verbeek et al., 1995). This block occurs at the ISP stage when small non-cycling cells accumulate resulting in a ten-to one hundred-fold reduction in numbers of DP and SP cells. As these Tcf-1 deficient mice age, thymocyte development deteriorates, resulting in additional blockades in development at the DN2 and DN4 stages (Schilham et al., 1998). These populations are usually characterised by extensive proliferation. In the Tcf-1 deficient mice DN2 cells are consistently absent and DN4 cells are absent (in mice over 4 weeks of age) or fail to proliferate. This result indicates that Tcf-1 functions in the control of an expansion of developing thymocytes. In addition, extrathymic development of intestinal γδ T cells and liver NK T cells is impaired in these mice (Ohteki et al., 1996). Disruption of the Lef-1 gene did not result in a defect in thymocyte development but Lef-1 deficient mice die postnatally due to developmental defects in several non-lymphoid organs (van Genderen et al., 1994). The lack of a thymic phenotype in the Lef-1 deficient mice indicated that Tcf-1 and Lef-1 might have partially redundant roles in T cell development. Mice lacking both of these transcription factors have been generated and this redundancy has been confirmed (Okamura et al., 1998). Thymocytes from these double-deficient mice, when grown in foetal thymic organ cultures (FTOCs), exhibit a partial block at the transition between DN2 and DN3 cells and are totally blocked at the transition from ISP to DP stage. In addition, mice lacking
Tcf-1 and Lef-1 show defects in the differentiation of paraxial mesoderm and form additional neural tubes, similar to mice deficient for Wnt3a (Galceran et al., 1999). The implications of Tcf-1 as a downstream effector of the Wnt/β-catenin signalling pathway for T cell development have still to be clarified.

The basic/leucine zipper (bZip) transcription factor CREB (the cAMP response element binding protein) is a key regulator of a large number of genes that contain a CRE consensus sequence in their upstream regulatory regions. This includes certain T cell specific genes, such as the TCR β enhancer (Anderson et al., 1989; Gottschalk and Leiden 1990), so implicating this factor in thymocyte development. Transgenic mice expressing a dominant negative form of CREB have been generated (Barton et al., 1996). Although thymocyte development is not perturbed in these mice and thymocyte cell number is normal, thymocytes and T cells show a proliferative defect characterised by a markedly decreased IL-2 production. These cells are also more susceptible to apoptosis and G1 cell cycle arrest following a number of stimuli. CREB deficient mice have also been generated (Rudolph et al., 1998) which die at birth. In these embryos overall thymic cellularity is severely reduced and thymocyte development is impaired with a general increase in DN and a decrease in DP thymocytes. So, CREB is another transcription factor of importance in foetal T cell development.

The basic helix-loop-helix (bHLH) transcription factors have also been implicated in the regulation of gene expression during B and T cell development. These bHLH genes are divided into those that encode for transcriptional activators (including the E proteins E12 and E47 and HEB) and those that encode for repressors (such as Hairy Enhancer of Split 1, Hes-1). As described below, bHLH activators appear to drive lineage commitment and differentiation whereas bHLH repressors are important in cellular proliferation. The E proteins were identified by virtue of their binding to E-box motifs (CAN-NTG) in the immunoglobulin K and μ intronic enhancers. Four members have been described (E12, E47 together forming E2A, E2-2, Hes-1 and HEB) which can all bind E-boxes as homodimers or heterodimers with other proteins.
Functional roles for these proteins in B cell development have been elucidated (for review see Bain and Murre 1998). The role of these proteins in T cell development will be briefly mentioned here. Functional E-boxes have been identified within the TCRα and β enhancers and the regulatory elements of the CD4 gene. E2A deficient mice display a five to ten fold reduction in thymocyte and splenic cellularity (Bain et al., 1997). Thymocyte development is partially arrested at the DN1 stage, resulting in an accumulation of these cells that are not yet committed to the T cell lineage. Interestingly, CD25+ DN committed thymocytes are almost completely lacking. DP cells are reduced by 50% in comparison to wild type mice. Development of both αβ and γδ T cells is reduced but NK development is unimpaired. Furthermore deficiency of E2A results in the development of T cell malignancies. HEB deficiency affects thymocyte development after the DN2 stage, resulting in impaired αβ T cell development but unaffected γδ T cell development (Barndt et al., 1999). HEB deficient mice show reduced numbers of DP cells and an increase in immature single positive CD8 cells. The introduction of an αβTCR transgene is unable to rescue HEB deficient DN and ISP cells to the DP stage, implicating that HEB functions downstream or independently of pre-TCR signalling.

The activities of the bHLH transcription factors, including E2A and HEB, are regulated by a group of proteins termed inhibitors of DNA binding (Ids 1-4) (reviewed in Norton et al., 1998). This family of four proteins act as dominant-negative antagonists of the bHLH proteins by binding to them and sequestering them from transcriptional activation. Despite expression within the thymus deficiencies of Id1, 2 or 3 have previously been shown to have no effect on thymocyte development. However, Id2 deficient mice lack NK cells, lymph nodes and Peyers patches, although the splenic architecture is normal, exhibiting T-cell and B-cell compartments and distinct germinal centres (Yokota et al., 1999). Work by Spits and colleagues has elucidated a role for Id proteins in lineage choice, acting in an opposing way to Notch signalling. Overexpression of Id3 in foetal thymic organ culture resulted in inhibition of T cell development but promotion of NK development (Heemskerk et al., 1997). In addition, expression of Id 3 at a later stage appeared to inhibit αβ T cell development but
promote γδ T cell development (Blom et al., 1999). These results are consistent with the idea that Id3, via an effect on Notch/bHLH signalling, is important in determining lineage choice in thymocyte development. E2A and HEB deficient mice show neonatal lethality so preventing the generation of E2A-HEB double mutants through simple genetic crossings. This would enable the level of functional redundancy between these two family members to be established. The generation of transgenic mice that overexpress Id1 within the thymus has recently allowed this, and the role of Id1 in the thymus, to be assessed (Kim et al., 1999a). The total number of thymocytes in these mice is less than 4% of that in wild-type mice. The majority of thymocytes present are DN with a block at the DN1 stage similar but more severe than the block in E2A deficient mice (Bain et al., 1997). Massive cell death is seen in the Id transgenics with 50% of the thymocytes undergoing apoptosis. Despite this high level of cell death Id1 transgenic mice develop T-cell lymphomas later in life, at various times in thymocyte development. Id1, through its interactions with the E proteins, is therefore essential for normal T cell differentiation and tumour suppression.

Another bHLH gene, Hes-1 is also implicated in early thymocyte development due to its expression in lymphoid tissues and due to being identified as a downstream target of the Notch signalling pathway (Conlon et al., 1995; Jarriault et al., 1995; Kageyama and Ohtsuka 1999). Hes-1 deficient mice die shortly after birth with defects of neural tube and eye morphogenesis (Ishibashi et al., 1995; Tomita et al., 1996). The majority (over 90%) of these mice completely lack the thymus or possess a much smaller thymus than wild-type mice (which lacks TCRβ and TCRγδ expression) so indicating that mature T cells are absent (Tomita et al., 1999). To further study the role for Hes-1 in thymocyte development foetal liver cells were injected into irradiated RAG-2 host mice to see if Hes-1 deficient thymocytes can develop when in the environment of normal thymic stroma (Tomita et al., 1999). In this system B cells, myeloid, macrophage and erythroid cells develop normally but mature T cells do not, with thymocyte development blocked within the DN compartment. Numbers of DN1 cells were comparable with wild-type mice but cell numbers at subsequent
stages were considerably reduced so elucidating a role for Hes-1 in the expansion of DN thymocytes (Tomita et al., 1999).

1.8.1. A brief introduction to the CBF transcription factors

The role of the previously mentioned transcription factors in commitment to the lymphoid lineage and/or role in thymocyte development is well established. Discussion will now turn to a recently identified family of heterodimeric transcription factors, the Core Binding Factor (CBF) family, whose role in T cell development is as yet unresolved. This is the subject for the second part of this thesis and is covered fully in Chapter 5.

The CBF family of heterodimeric proteins are comprised of two unrelated subunits, a DNA binding α subunit and a non DNA-binding β subunit (Kamachi et al., 1990; Wang and Speck 1992; Ogawa et al., 1993b; Wang et al., 1993). The α subunit is encoded by three distinct genes, while only one gene is known to code for the β subunit in mammals (Bae et al., 1993; Ogawa et al., 1993b; Levanon et al., 1994; Bae et al., 1995). The α subunits contain a conserved 128 amino acid DNA binding domain, known as the Runt domain because of its high homology to the Drosophila pair-rule gene runt (Kania et al., 1990). CBF and RUNT proteins bind the enhancer core sequence TGYGGT. This sequence motif is present in the transcriptional enhancers of many T cell specific genes, including the enhancers of the TCR α, β, γ and δ chain genes as well as numerous other haematopoietic specific genes (for details see Chapter 5 and Speck and Terryl 1995). The activation of numerous haematopoietic promoters by CBF implies a role for this family of transcription factors in haematopoietic development. This is further evidenced by the fact that the CBFα2 and CBFβ genes are disrupted by consistent chromosomal translocations associated with haematopoietic malignancies. In addition gene disruption experiments in mice have demonstrated that both CBFα2 and CBFβ are required for haematopoietic development. CBFα2 and CBFβ homozygous deficient mice die between embryonic days 11.5 and 12.5 due to haemorrhaging in the CNS (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997) and definitive haematopoiesis (erythropoiesis and myelopoiesis) in the liver was severely impaired. CBFα1 deficient mice have also been generated. These mice die of respiratory failure shortly after birth due to the absence of mature osteoblasts
and a complete lack of bone formation (Komori et al., 1997; Otto et al., 1997). Thymocyte development was normal in the \textit{CBF\alpha1} deficient mice, suggesting that other family members are involved or that the role of CBF\alpha1 in thymopoiesis is redundant. The early lethality of the \textit{CBF\alpha2} and \textit{CBF\beta} deficient mice precluded an investigation of the role of the CBF transcription factors in thymocyte development. The requirement for CBF in the transcriptional activation of numerous T cell specific genes and the expression of the CBF proteins throughout development, however, indicates a function for these factors in thymocytes (Meyers et al., 1993). It is clear from the above discussion how investigations of the molecular details of thymocyte development have benefited from gene targeting and transgenic studies. The aim of the second part of my thesis is to investigate the nature of the role of CBF proteins in T cell development, using a transgenic approach.
Chapter 2

Materials and Methods

2.1. Buffers, Solutions and Bacteriological media

All chemicals were of analytical grade or equivalent and were obtained from Sigma, UK or BDH, UK. All buffers and solutions were made in deionised water and sterilised by autoclaving or filtration where appropriate.

**SSC (20x stock):** 3M NaCl, 0.3M NaH$_5$Na$_3$O$_7$, adjust to pH 7 with 1M NaOH

**TAE (50x stock):** 2M Tris base pH 8.0, 1M Glacial acetic acid, 50mM EDTA pH 8.0

**TE:** 10mM Tris base pH 7.4, 1mM EDTA pH 8.0

**PBSA (pH 7.4):** 137mM NaCl, 3.3mM KCl, 1.7mM KH$_2$PO$_4$, 10mM Na$_2$HPO$_4$, adjust to pH 7.4 with HCl

The following bacteriological media were obtained from ICRF Central Services:

**L– broth (LB):** 0.5% bacto-yeast extract, 1% bacto-tryptone, 1% sodium chloride adjusted to pH 7.6 with 10M potassium hydroxide

**L-agar:** LB, 15g/L bacto-agar.

2.2. Nucleic Acids – DNA

2.2.1. Small scale preparation of plasmid DNA ('minipreps')

For preparations from plasmids, single ampicillin resistant colonies were picked into 5ml LB plus appropriate antibiotic selection and shaken overnight at 37°C. 1.5ml of this culture was centrifuged for 3 minutes at 13000rpm. Bacterial pellets were resuspended in 100μl of Solution I, followed by addition of 200μl of
Solution II and incubation at 4°C for 5 minutes. 150µl Solution III was added to the lysate which was then vortexed vigorously, incubated for additional 5 minutes at 4°C and centrifuged for 10 minutes at 13000rpm. The supernatant was transferred to another tube and DNA was precipitated with 0.1 volumes 3M sodium acetate and 2 volumes of ice-cold absolute ethanol, pelleted and washed once in 70% ethanol, before being resuspended in deionised water.

2.2.2. Large scale preparation of plasmid DNA ('maxipreps')

For maxipreps from plasmids, 1ml of a miniprep culture was added to 2 times 400ml LB containing 50 µg/ml ampicillin and shaken overnight. Bacteria were pelleted by centrifugation at 5000rpm and resuspended in 30ml of Solution I, followed by 45ml of Solution II and 45ml of Solution III. The lysate was separated from cell debris by centrifuging at 5000rpm for 15 minutes at 4°C and the supernatant filtered into a clean bottle. The plasmid DNA was precipitated from this supernatant by the addition of 0.8 volumes of isopropanol and spinning at 8000rpm for 30 minutes at room temperature. The DNA pellet was resuspended in 50mM TE pH 8.0 to a final volume of 9ml. 1.05g of caesium chloride per ml and 500µl of 10mg/ml ethidium bromide were added to the solution. DNA was isolated after spinning at 45000 rpm in a Beckman ultracentrifuge for 24 hours. Supercoiled DNA bands were removed by aspiration and ethidium bromide extracted with several changes of caesium chloride saturated isopropanol. The DNA was then precipitated by the addition of 0.1 volumes sodium acetate and 2 volumes of absolute ethanol and centrifugation at 8000rpm at room temperature for 15 minutes. Finally, the DNA pellet was resuspended in deionised water and the concentration and purity determined by spectroscopy. Maxiprep DNA was aliquoted and stored at -20°C.

2.2.2A. Solutions for plasmid preparation

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<tr>
<td>50mM glucose</td>
<td>3.6g glucose</td>
</tr>
<tr>
<td>10mM EDTA pH 8.0</td>
<td>8ml 0.5M EDTA</td>
</tr>
<tr>
<td>25mM Tris- HCl pH 8.0</td>
<td>10ml Tris (pH 8.0)</td>
</tr>
</tbody>
</table>
Final Per 100ml

**Solution II:**
0.2M NaOH 0.8g NaOH
1% SDS 1.0g SDS

Per 100ml

**Solution III:**
3M potassium 60ml potassium acetate
5M acetate (pH 4.8) 11.5ml glacial acetic acid

### 2.2.3 Estimation of nucleic acid concentration

For concentrations of DNA thought to be in excess of 300ng/µl the sample was diluted in distilled water by a factor of 500 and placed in a quartz cuvette. The optical density (OD) of the solution was measured, relative to water, at a wavelength of 260nm on an LKB spectrophotometer. The nucleic acid concentration, in µg/ml, was calculated by multiplying the absorbance reading by the dilution factor and then by 50 for DNA, 40 for RNA and 33 for oligonucleotides. In order to check the purity of the sample, the OD at 280nm was also measured. A ratio $(\text{OD}_{260}/\text{OD}_{280})$ of 1.7 - 1.95 indicated a protein free sample. For samples less than 300ng/µl 1-2µl of DNA was run on a 1% agarose/TAE ethidium bromide stained gel alongside standards of known concentration. The concentration of the sample was then estimated by comparing the intensity of its fluorescence to that of the standards under UV light.

### 2.2.4 Restriction enzyme digestion

DNA digestion with restriction enzymes were performed using the optimal conditions as recommended by the particular manufacturer (Boehringer or New England). Reactions were performed using 1x buffer, DNA (10ng – 1µg/µl) and 0.5 – 5U of enzyme per µg DNA. The total volume of enzyme used was always less than 10% of the reaction volume to avoid glycerol inhibition of digestion. Incubations were at 37°C, unless otherwise instructed, for periods from 1 hour to overnight.
2.2.5. Polymerase chain reaction (PCR)

PCR was used to amplify cloned, genomic or cDNA. Short (21 mer) oligonucleotide primers (ICRF) were designed to have roughly 50% G/C content. Template DNA was mixed with 100ng of sense and antisense primers, dATP, dCTP, dGTP and dTTP each at 200 μM, 5 μl of cDNA sample or 200ng of genomic DNA in a final concentration of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl2, 0.1% Triton X-100, and 2.5 units of PiTaq polymerase (in house) in a 0.2 ml Eppendorf tube. Amplification was carried out in a Peltier thermal cycler (MJ Research). Standard PCR conditions were as follows:

First cycle: denaturation at 94 °C for 4 minutes
annealing at Tm of primers for 30sec*
extension at 72 °C for 1 minute per Kb

Subsequent 29 cycles: denaturation at 94 °C for 20 secs
annealing at Tm of primers for 30secs min*
extension at 72 °C for 1 minute per kb

Final extension period: 72°C for 5 minutes

*Where Tm = n(A + T)x 2 + n(C + G) x 4

After PCR, 1/10 of each reaction product was visualised on an agarose gel. If the product was required for further manipulation, contaminating proteins were removed by treatment with 10mg/ml proteinase K (Sigma) in DNA Lysis buffer for 1 hour at 56°C, followed by phenol/chloroform extraction and ethanol precipitation.

2.2.6. DNA fragment purification

All DNA fragments for either cloning or DNA probes were isolated using Wizard DNA Clean-Up System (Promega) or Jetsorb (Genomid).

2.2.6A. Wizard DNA Clean-Up System

DNA was cut and modified with appropriate enzymes and run slowly on a 0.7-2% TAE gel. The band of interest was excised from the gel by using a hand held short wave UV lamp and scalpel. The gel slice was placed in a PCR tube half fill with glass wool with a needle hole in the bottom, which was placed in a 1.5ml
Eppendorf tube with the lid cut off. This was centrifuged for 10 minutes and then 100 µl of water added and the spin repeated. To the eluant 1ml of Wizard DNA Resin was added and mixed (1ml resin per µg DNA). The DNA/Resin mix was loaded onto a Wizard column on a vacuum manifold and the resin pulled through the column. Then 2ml of 80% isopropanol was added and drawn through the column. The column was removed and centrifuged in an Eppendorf tube without a lid for 20 seconds then placed at 37°C for 5 minutes to remove any residual isopropanol. 40µl of hot water (60-70°C) was added to the column and left for 60 seconds. The column was centrifuged for 30 seconds and the eluant removed to a clean tube. The DNA was now ready for further manipulation.

### 2.2.6B Jetsorb system

The band of interest was excised from the gel and placed in an Eppendorf tube with 300 µl of buffer A1 and 10µl JETSORB suspension per 100mg of gel slice. The mixture was vortexed and incubated at 50°C for 15 minutes with mixing every 3 minutes. The mixture was centrifuged and the supernatant removed. The pellet was washed with 300µl A1 buffer, centrifuged and the supernatant removed. The pellet was washed twice in 300µl A2 buffer, centrifuged and the supernatant removed. The pellet was dried, resuspended in 20µl of water incubated for 5 minutes at 50°C, centrifuged, and the eluant removed to a clean tube. The DNA was now ready for further manipulation.

### 2.2.7. Oligo - labelling of DNA probes

Fragments of 100-500 base pairs were used as probes. Oligo labelling of DNA relies on the ability of random hexanucleotides to anneal to multiple sites along the length of a DNA template. The primer-template complexes formed represent a substrate for the Klenow fragment of DNA polymerase. OLB was made by mixing solutions A, B and C in proportions 10:25:15 respectively and stored at -20°C. 100ng of DNA was denatured by boiling for 5 minutes. The DNA was placed on ice for 2 minutes to prevent re-annealing and the following reagents added: 10µl OLB, 1µl 10mg/ml BSA, 5µl [³²P]-dCTP 3000Ci/mmol (Amersham), and 10U of Klenow fragment of DNA polymerase I, in a total
volume of 50μl. After overnight incubation at room temperature, the reaction was centrifuged at 1500rpm for 5 minutes through a Sephadex G-50 medium grade column equilibrated in 3x SSC, to remove unincorporated label. The eluate was counted in an Oncor Probecount to determine specific activity. The probe was then boiled for 5 minutes, placed on ice for a further 2 minutes prior to hybridisation. Probes used were routinely of specific activity of >10^8 dpm/μg DNA.

2.2.7A. Reagents used to prepare oligonucleotide labelling buffer (OLB)

Solution A: 1.25M Tris-HCl pH 8.0, 0.125M MgCl₂
18 μl β-ME per ml
5 μl per ml each of 0.1M dATP, dTTP, dGTP in TE (Pharmacia)

Solution B: 2M HEPES pH 6.6

Solution C: 90 OD units/ml random hexadeoxy-ribonucleotides (Pharmacia)

Alternatively, the Stratagene Prime It II random primer kit was used. This kit is designed to produce high-specific-activity DNA probes within 2-10 minutes, so the overnight incubation step can be avoided. Manufacturers protocol was followed.

2.2.8. Preparation of genomic DNA

Mouse tail biopsies were placed immediately into 750μl DNA Lysis buffer. 2.5μl Proteinase K (10mg/ml) was added and incubated overnight at 56°C. The lysate was then extracted with an equal volume of phenol/chloroform/isoamyl alcohol to remove denatured proteins. DNA was precipitated with 750μl of isopropanol, recovered by centrifugation in an Eppendorf, centrifuged for 10 minutes at 14000rpm, washed once in 70% ethanol, and left to dissolve in deionised water at 56°C for one hour.
2.2.8A Solutions for genomic DNA preparation

**DNA Lysis Buffer:** 100mM sodium chloride, adjusted to pH 8.0 with 10M NaOH, 10mM Tris-HCl, 0.1% SDS, 1mM EDTA pH 8.0

**Phenol/chloroform solution:** Tris equilibrated phenol/0.1% 8-hydroxyquinoline was added to an equal volume of a solution of chloroform: isoamyl alcohol (24:1).

2.2.9. Southern blot analysis of genomic DNA

10μg genomic DNA was digested overnight in a total volume of 30μl together with 30 units of the appropriate restriction enzyme, thereafter the samples were electrophoresed at 1.5V/cm overnight on a 0.7% TAE agarose gel. The gel tank was washed out prior to use and the running buffer was freshly prepared. Digested samples were loaded onto gels using fresh loading buffer to avoid any potential plasmid contamination. The gel was photographed with a ruler beside it, next to the DNA marker lane. To improve the efficiency of transfer of large restriction fragments from the gel to the membrane, the gel was placed in depurination solution for 15 minutes. Mild acid treatment of large restriction fragments facilitates their cleavage to smaller fragments that are more readily transferred from the gel to the membrane. After depurination, the gel was rinsed briefly in deionised water, agitated in excess denaturing solution for 30 minutes and then transferred to nitrocellulose membrane (Hybond N') in denaturation solution by capillary action overnight. The inverted gel was placed onto a wick comprised of a piece of Whatman 3MM paper dipped into a tray of denaturing solution. The following were placed in order on top of the gel: 1 piece of nylon membrane soaked initially in water and then in denaturing solution for 20 minutes, 3 denaturing solution soaked 3MM paper, 20-40 sheets of dry 3MM paper, a 5cm stack of dry paper towels, a glass plate and a weight of less than 0.5Kg. This blotting set up was left for 12-16 hours. The apparatus was dismantled and the position of the wells were marked on the filter in a...
waterproof pen. The blot was rinsed in 2x SSC and UV cross-linked to immobilise the DNA (Stratagene UV crosslinker).

2.2.9A Solutions for Southern blotting:
Depurination solution: 0.25M HCl

Denaturing solution: 1.5M NaCl, 0.5M NaOH

2.2.10. Hybridisation of Southern blots

Hybridisation reactions took place within pyrex glass roller bottles which were constantly rotated within an oven (Hybaid) equilibrated to the appropriate temperature. The filter was pre-hybridised in 10ml of Church buffer for 2-6 hours at 65°C. The boiled, labelled probe was then added to the Church hybridisation buffer. Hybridisation of the probe to the filter was performed overnight at 65°C. After hybridisation the filter was washed using varying levels of stringency. For low stringency washing of blots hybridised with cross-species probes, a solution of 3x SSC/0.1% SDS was used at 55°C. After each individual wash, blots were monitored to prevent complete removal of signal. For high stringency washing of blots two washes with 2x SSC/0.1% SDS were performed at 65°C for 15 minutes each, followed by a single 15 minute wash with 0.1x SSC/0.1% SDS at 65°C. Blots were exposed at -70C on Kodak XAR5 film using intensifying screens.

Church Hybridisation Solution: 0.5M NaH₂PO₄, 7% SDS, 1mM EDTA pH8.0

2.2.11. Membrane Stripping

A solution of 0.1%(w/v) SDS was pre-boiled and poured over wet blots. The blots were incubated with gentle agitation until the solution had cooled. Probe removal was confirmed by autoradiography.
2.3. Nucleic Acids – RNA

2.3.1. Total RNA isolation

For the preparation of RNA gloves, sterile plastic and fresh deionised water and autoclaved solutions were used and the samples were kept at 4°C or below at all times. Total RNA was prepared using the TRIzol reagent (GibcoBRL) and the instructions recommended by the manufacturer. TRIzol consists of a mono-phasic solution of phenol and guanidine isothiocyanate. Cells were washed once with PBS and then resuspended in 1ml of Trizol reagent. The lysate was passed several times through a pipette and incubated at room temperature for 10 minutes to allow complete dissociation of nucleoprotein complexes. 0.2ml of chloroform was added and the samples were mixed and incubated at room temperature for a further minute. The samples were then centrifuged for 15 minutes at 4°C at 13000 rpm. Following centrifugation, the RNA-containing aqueous phase was transferred to a fresh tube and precipitated by the addition and mixing of 0.5ml of isopropyl alcohol. If using less than 50000 cells 200µg glycogen was added to act as a carrier. RNA was allowed to precipitate for 20 minutes at room temperature and then centrifuged for 15 minutes at 13000 rpm. The resulting pellet was washed with the addition of 1ml of 70% ethanol and then re-centrifuged for 5 minutes at 13000 rpm. Pellets were finally dissolved in 20-100µl deionised water and stored at -70°C until required. RNA purity was assessed by the ratio OD 260/280, with a ratio of 1.8 or greater being suitable for subsequent downstream manipulation.

2.3.2. RNA agarose gels for Northern Blotting

To avoid degradation of the RNA the gel tank and the gel tray were first washed with 0.1M NaOH and rinsed well in deionised water. 1.5g agarose was boiled in 130ml deionised water, after cooling to ~60°C 15ml of 10x MOPS and 7.6ml formaldehyde (Fluka) were added and the gels was poured into a prepared gel tray in the fume cupboard. RNA samples (5-20µg) were made up to 4.5µl in deionised water. To this 2 µl 10x MOPS, 3.5 µl of 37% formaldehyde and 10µl of 100% formamide were added and mixed well. The samples were then incubated at 65°C for 5-15 minutes before being placed on ice. Before
loading the sample onto the gel 8μl of loading buffer was added and the wells of
the gel were rinsed out with running buffer. 10μl RNA ladder (0.24-9.5Kb, GibcoBRL) was also loaded. The samples were run through the gel at 1-5V/cm
in 1x MOPS buffer. The gel was photographed with a ruler beside it and then
soaked in 10x SSC to wash away formaldehyde for 2 x 20 minutes. The gel was
then blotted onto 20x SSC soaked nitro-cellulose Hybond N (Amersham) filters
in the manner described for Southern blot transfer. The blot was rinsed in 10x
SSC and RNA immobilised by UV crosslinking.

2.3.2A. Solutions for Northern blotting
10x MOPS: 0.2M MOPS, 50mM Na acetate, 10mM EDTA

6x loading buffer: 0.25% bromophenol blue, 0.25% xylene-cyanole
30% glycerol

2.3.3. Membrane hybridisation for Northern blots

RNA filters were pre-wetted in 2x SSC and placed in pyrex bottles
(Hybaid). The filters were prehybridised for a minimum of 2 hours at 42°C
rotating in 20ml of RNA hybridisation solution without probe. The buffer was
replaced and prehybridisation repeated for an additional 2 hours. The preboiled
32P labelled probe was then added to 15ml fresh RNA hybridisation solution
also containing salmon sperm DNA (50μg/ml final concentration; Sigma)
Hybridisation took place over night, at 42°C in rotating pyrex bottles. After
hybridisation the blot was washed twice for 20 minutes in 2x SSC, 0.1% SDS at
room temperature to remove unbound surface probe and then given two
washes of 0.1x SSC, 0.1% SDS at 52°C for 20 minutes with shaking. The blot was
briefly dried on 3MM to remove excess fluid and than wrapped into plastic film
(Saranwrap) and subject to autoradiography or placed on the Molecular
Dynamics PhosphorImager and quantitated via accompanying ImageQuant
software (Molecular Dynamics, Sunnyvale, CA).
2.3.3A. Solutions for hybridisation of Northern Blots

Pre-/hybridisation solution: 5x SSC, 1% SDS, 5x Denhardt’s solution
60% formamide, 10mg/ml poly A (Sigma)
7% Dextran Sulphate (Pharmacia)
20mM Na phosphate buffer (pH 6.8)
100 μg/ml tRNA (bakers yeast (Sigma))
100 μg/ml fresh denatured and sheared salmon sperm DNA (Sigma)

5x Denhardt’s solution: 0.1% Ficoll (Type 400, Pharmacia)
0.1% Polyvinylpyrrolidone (Sigma)
0.1% BSA (fraction V, Sigma)

2.3.3B. Details of probes used for Northern blots

Details of the various probes used for hybridisation of Northern blots are
given in the following sections: section 3.2.2 describes the generation of murine
\( \beta\)-Actin and p56\(^{\text{leck}} \) specific probes, sections 4.2.2 and 5.2.1A give details of probes
specific for GFP transcripts and for RUNT-containing transcripts respectively. In
addition a probe specific for murine GAPDH was obtained via Not1/SalI
digestion of full length GAPDH cDNA previously cloned into pSPORTs,
resulting in a 1.2Kb probe.

2.3.4. Reverse Transcriptase PCR

Reverse transcription of RNA and PCR amplification of the resulting
cDNA fragments (see above) was used to analyse the presence of a number
endogenous and transgenic transcripts in various mouse tissues. First the RNA
was treated with DNase (RQ1 RNase-free DNase, Promega) to remove any
DNA contamination for 30 minutes at 37°C. Following this, two
phenol/chloroform plus one chloroform extractions were performed. The RNA
was precipitated with 0.1 volume 3M sodium acetate and 2 volumes of absolute
ethanol and 200μg glycogen was added to act as a carrier. cDNA synthesis was
carried out using the superscript preamplification system (GibcoBRL). The first
strand cDNA synthesis reaction is catalysed by Superscript II RNase H- reverse
transcriptase (RT). This enzyme has been engineered to eliminate the RNase-H
activity found in other RTs that degrades the RNA in the first strand reaction.
Manufacturer's protocol was followed. In brief, following resuspension in 10.5μl of RNase-free water and addition of 50ng of random hexamers the RNA was heat denatured for 10 minutes at 70°C and then cooled on ice. The reverse transcription (RT) reaction was carried out in the presence of 0.5μl RNasin (GibcoBRL), 20mM Tris-HCl (pH 8.4), 50mMKCl, 2.5mM MgCl2, 0.5mM mixed dNTPs, 10mM DTT using 200U Superscript II RT. Following incubation at 42°C for 50 minutes the reaction was terminated at 70°C for 10 minutes and chilled on ice. In general the cDNA volume was made up to 100μl before use in PCR reactions. The PCR reactions were carried out in a 50μl reaction volume, unless otherwise stated.

2.4. Protein

2.4.1. Protein purification - Total cell extracts

A single cell suspension from tissues or following cell sorting was first washed in ice cold PBS. The cells were pelleted by centrifugation at 1200rpm for 5 minutes at 4°C and the supernatant removed. The cell pellet was resuspended in 100μl Triton lysis buffer and incubated on ice for 15 minutes, vortexing gently every 3 minutes. The lysates were centrifuged at 13000rpm for 15 minutes. The supernatants were then spun through microconcentrator columns (Millipore, Bedford MA) to concentrate the proteins in each sample. 25μl of SDS sample buffer was then added to each sample before separation by electrophoresis on 8% SDS-polyacrylamide gels (SDS-PAGE).

1% Triton lysis buffer: 50mM Tris (pH 8), 20% Glycerol
150mM NaCl, 1% Triton X 100
100μg/ml PMSF (added fresh)
Protease inhibitors (1μg/ml, added fresh)

2.4.2. SDS-polyacrylamide gel electrophoresis

The separation of proteins was carried out using SDS-PAGE. Two glass plates (18x16 cm) were assembled with 1.5 mm spacers and clamped together in a protein gel apparatus. Two layers of parafilm were placed beneath the plates, before clamping to ensure tight seal. 30ml of separating gel mix was prepared
just before use and poured into a vertical gel apparatus (Hoefer), avoiding air bubbles and leaving ~3cm gap for the stacking gel and the comb.

<table>
<thead>
<tr>
<th>Reagents:</th>
<th>10%</th>
<th>12%</th>
<th>15%</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide (37.5:1)</td>
<td>10ml</td>
<td>12ml</td>
<td>15ml</td>
<td>1.7ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>14ml</td>
<td>12ml</td>
<td>9ml</td>
<td>5.8ml</td>
</tr>
<tr>
<td>2M Tris pH 8.8</td>
<td>5.64ml</td>
<td>5.64ml</td>
<td>5.64ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris pH 6.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.5ml</td>
</tr>
<tr>
<td>20% SDS</td>
<td>150µl</td>
<td>150µl</td>
<td>150µl</td>
<td>125µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>30µl</td>
<td>30µl</td>
<td>30µl</td>
<td>10µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>300µl</td>
<td>300µl</td>
<td>300µl</td>
<td>100µl</td>
</tr>
</tbody>
</table>

The mixture was overlaid with 0.25% SDS. After the gel had set the stacking gel was poured and a comb inserted. The protein samples were prepared by addition of half a volume of sample buffer followed by boiling for 5 minutes. 15µl of Rainbow markers (Amersham) were also loaded as protein standards. The gel was run at 20mA until the samples had passed through the stacking gel and then at 30mA for approximately 4-5 hours until the markers had separated sufficiently and the blue dye front had reached the bottom of the gel.

2.4.2A. Solutions for SDS-PAGE

4x sample buffer: 62.5 mM Tris-HCl, pH 6.8, 10% Glycerol, 2% SDS, 5% β-ME, 0.05% bromophenol blue

1x running buffer: 3g/L Tris, 14.4 g/L Glycine, 1g/L SDS (5ml 20% SDS)

2.4.3. Western Blotting

SDS-PAGE gels were transferred using a Hoefer Semi-Phor blotting apparatus. The stacking gel was removed and the size of the separating gel measured. Six pieces of blotting paper and a piece of Immobilon™-P PVDF 0.45µm membrane were cut to the size of the gel. The membrane was pre-wet in methanol, rinsed with deionised water and then soaked with the blotting paper in transfer buffer for 2 minutes. Transfer buffer was 10mM CAPS pH 11.0. A mylar mask with an opening 2mm smaller than the gel in width and length was placed over the electrode. Three sheets of blotting paper, followed by the
membrane, then the gel and three more pieces of blotting paper were centred
over the opening in the mylar mask and the edges rolled out ensuring there
were no air bubbles which would block the transfer of proteins. The proteins in
the gel were transferred onto the membrane using a current of 0.8mA/cm² of gel
for 45 minutes. The blot was removed and marked to identify on which side the
proteins were transferred, the coloured marker proteins gave an indication of
the efficiency the transfer.

The membrane was rinsed once with water and placed into blocking
solution for 5-10 minutes and incubated with the primary antibody in blocking
solution, over night on an orbital shaker at 4°C. Excess primary antibody was
removed by washing the membrane 3 times for 10 minutes in wash buffer. The
Western blot was then incubated with a secondary antibody, coupled with
horseradish peroxidase (HRP), in 10ml blocking solution for 30 minutes at room
temperature on an orbital shaker. The membrane was washed 3x in wash buffer
and rinsed one with water to remove residual milk powder from the wash
buffer. The protein-antibody complexes were detected with an ECL-kit
(Amersham).

2.4.3A. Solutions for Western Blotting

1x TNT (pH 7.6): 36g/4L NaCl, 4.8g/4L Tris, 20ml/4L Tween 20

wash buffer: 1% milk powder in 1x TNT

blocking buffer: 5% milk powder in 1x TNT

2.5. Cloning

2.5.1. Dephosphorylation of vector

Calf intestinal phosphatase (CIP) treatment was employed to reduce the
possibility of vector self ligation. After restriction digestion, vector DNA was
diluted to 50µg/ml and mixed with 1 unit of CIP (Boehringer) and 1x CIP buffer
for 1 hour. Vectors with blunt end or 3' overhanging ends were incubated with
CIP at 50°C, whereas 5' overhanging ends were treated with CIP at 37°C. The
CIP reaction was terminated (and any remaining proteins destroyed) by
treatment with 2µl proteinase K (10mg/ml) in the appropriate buffer for 30
minutes at 56°C. Following one phenol chloroform extraction and ethanol precipitation the DNA was resuspended in 25μl distilled water.

**Proteinase K buffer**  100mM Tris (pH8), 200mM NaCl, 5mM EDTA, 0.2% SDS

### 2.5.2. End-Filling with Klenow DNA Polymerase I

DNA (2-6 μg) which had been cut with suitable restriction enzymes was end-filled with 4U Klenow DNA Polymerase I (Boehringer) in a volume of 50-100μl (so DNA to be filled was at a final concentration of 50μg/ml) and a final concentration of 33-66 μM dNTPs and in the presence of a 1x concentration of a standard MgCl₂ containing restriction buffer. The mixture was incubated at room temperature for 30 minutes and the reaction stopped by adding EDTA to a 10mM final concentration and heating at 75°C for 10 minutes.

### 2.5.3. Glycerol stocks of bacteria.

In a Nunc 1.5ml freezing tube 0.85ml of an overnight culture was mixed with 0.15 ml of glycerol and frozen on dry ice. Glycerol stocks were stored at -70°C.

### 2.5.4. Ligation of DNA fragments into plasmid cloning vectors

The gel-purified vector and insert were set up in a ligation reaction as follows:

**Ligation mixture:**  Ypl vector (40ng), 1 μl 10 x Low Salt Buffer

1μl 10 x Ligation Additions Buffer

Xμl purified insert

1μl T4 DNA ligase (100 units/μl)

distilled water to 10μl final

Different ratios of insert:vector were set up, generally 1:1, 3:1, 10:1. The ligations were incubated at room temperature for 30 minutes-3 hours. The ligase was heat
inactivated at 65°C for 10 minutes and 2-5μl was transformed into competent bacteria.

10 x Ligation additions buffer

10 x Low salt Buffer

These buffers were aliquoted and stored at -20°C.

2.5.5. Double blunt-end ligations.

Vector DNA (2-3μg) and insert DNA (9μg) were cut with the appropriate restriction enzymes in volumes of 40μl at 37°C for 2-3 hours. The enzymes were heat inactivated at 75°C for 10 minutes. The vector and insert were both end filled with 8U Klenow DNA Polymerase I, with dNTPs at a final concentration of 33-66μM in 60μl with 1x restriction buffer. The Klenow was heat inactivated by adding 1μl of 0.5M EDTA and heating at 75°C for 10 minutes. The vector and insert DNA were then run slowly on a 0.7% TAE agarose gel. The bands of interest were excised from the gel with a clean scalpel and the DNA purified using JETSORB in a final elution volume of 20μl. To check the DNA concentration, 2-4μl were run on a gel. Ligation reactions were set up as above, but using 80-120ng of insert and vector ratios in the range of 3:1, 12:1 and 24:1. Ligations were performed at 16°C overnight in the cold room. Supercompetent bugs (10^6-10^7cfu/ml) were transformed with 2-5μl of the ligation reactions. Recombinants were found by either colony hybridisation or picking up to 40 colonies and minipreping them.

2.5.6. Preparation of Electrocompetent bacteria.

A fresh plate of XL-1 colonies was prepared by streaking a sample from a glycerol stock onto an LB-tetracycline plate and incubating overnight at 37°C. 20ml of warm LB was inoculated with three fresh colonies and grown at 37°C in a 100ml flask with vigorous shaking. When an OD595 of 0.2 had been reached 80ml of warm LB were added and the culture grown again to OD595 of 0.2.
Finally 250ml of warm LB were added and the culture split into two 1 litre flasks and grown to OD595 of 0.5. The culture was placed on ice for 5 minutes and then centrifuged in a cold Beckman centrifuge JA14 rotor for 10 minutes to pellet the cells. All proceeding steps were performed on ice. The cells were resuspended and washed in 1 volume of cold fresh Millipore water, centrifuged at 3000rpm for 10 minutes, washed in 0.5 volume of cold Millipore water and centrifuged again. The pellet was resuspended in 20ml of cold fresh Millipore water with 10% glycerol and placed in two 50ml Falcon tubes. These were centrifuged at 3000rpm and the pellets resuspended in 0.5 times the pellet volume of Millipore water with 10% glycerol. Cells were divided into 100μl aliquots on dry ice and placed at -70°C.

2.5.7. Electroporation of competent bacteria.

Electroporation was carried out using a Bio-Rad E.Coli Pulser. DNA (1-4μl) was mixed with 40μl of cells on ice for 1 minute, and then transferred to a 0.1cm cuvette. Electroporation conditions were as follows: pulse 1.8kV, Resistance 200 ohms, Capacitance 25μF. A time constant between 4.5-5 was considered optimal. Following the pulse 1ml of LB was added and the culture shaken for 1 hour at 37°C. Various volumes of the culture were plated on LB - Ampicillin plates.

2.6. Cell Biology Procedures

2.6.1. Immunofluorescence analysis by flow cytometry

Single cell suspensions from thymus, spleen and lymph nodes were prepared by gently disaggregating the tissues on a piece of gauze with a syringe plunger in FACS buffer (PBS with 5% FCS). Cell suspensions were filtered and were stained for the detection of surface antigens and then analysed by flow cytometry. For the majority of FACS analysis, 10⁶ cells were stained per sample. Antibodies (Pharmingen or Caltag) were generally diluted 1 in 100 in FACS buffer. Antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used at a final concentration of 5μg/ml. Alternatively antibodies conjugated with biotin were used followed by second layer of
0.02µg/ml streptavidin-Tricolour (Caltag). These fluorochromes are excited by a 488nm laser, with emission wavelengths of 525, 575 and 667nm respectively.

All incubations for FACS analysis were performed at 4°C. For staining, the appropriate number of cells were aliquoted into individual FACS tubes and pelleted by centrifugation at 1200rpm for 5 minutes. The pellet was resuspended in 100µl of FACS buffer together with a 1:100 dilution of the relevant antibody and incubated for 20 minutes on ice in the dark. Cells were then washed free of unbound antibody by the addition of 2ml of FACS buffer, centrifuged at 1200rpm for 5 minutes and finally resuspended in 300µl of FACS buffer. If biotinylated primary antibodies were used cells from the primary stain were washed as above but resuspended in 100µl of FACS buffer together with a 1 in 100 dilution of Streptavidin-Tricolour, incubated for a further 20 minutes on ice in the dark and then finally washed and resuspended in 300µl of FACS buffer. Acquisition and analysis of stained cells by was performed on FACScan or FACSCalibur (Becton Dickinson), using Cellquest software. For routine staining, live cells were gated on forward and side scatter on a linear scale, and data for other parameters was collected in logarithmic scale. Unstained cells and cells stained with a single fluorochrome were used to set up the electronic gating collection parameters. In general, 1-5x10^5 events were collected per sample. All events were saved and gates were introduced to allow analysis of specific cell populations.

2.6.2. Double Negative enrichment

For analysis of the double negative thymocyte populations (CD44+CD25-DN1, CD44+CD25+ DN2, CD44-CD25+ DN3 and CD44-CD25- DN4) which constitute less than 3% of a wild-type mouse thymus, a technique can be used to enrich for these populations. This involves depletion of cells expressing CD4 and/or CD8 via complement lysis. Thymocytes were incubated in DMEM with supernatants from RL172.4 (anti-CD4) and 3.168.8.1 (anti-CD8) cells for 10 minutes at 37°C, rabbit complement (Low Tox-M Rabbit complement; Cedarlane, VH Bio Ltd,UK) was added and the cells were incubated for further 45 minutes at 37°C, gently mixing every 15 minutes. The living cells were separated from the lysed cells by Ficoll density-gradient centrifugation. Recovered cells were filtered and stained as follows: for DN1 cells were stained
with 1/50 dilution of biotinylated anti-CD117, 1/200 dilution of PE-anti-CD44 and 1/150 dilution of FITC-anti-CD25 followed by a second layer of 1/100 dilution of Streptavidin-Tricolour. The CD44+CD25-CD117+ DN1 cells can then be sorted if required. CD44+CD25-DN2 and the CD44-CD25+DN3 cells were analysed and sorted via staining, with 1/200 dilution of PE-anti-CD44 and 1/200 dilution of biotinylated anti-CD25 followed by a second layer of 1/100 dilution of Streptavidin-Tricolour. DN4 cells were stained with 1/100 dilution of PE-anti-CD2, PE-anti-Thy1.2, biotinylated anti-mouse γδT cell receptor (GL31), Tricolor conjugated CD8, Tricolor conjugated CD4 and 1/150 dilution of biotinylated anti-CD44 and anti-CD25 again followed by a second layer of 1/100 dilution of Streptavidin-Tricolour. The PE positive and Tricolour negative cells of DN4 population can then be sorted safely without contamination with non-thymocytes, γδ T cells or any CD4+ or CD8+ cells that may have survived depletion.

2.6.3. Cell Sorting

Single cell suspensions from thymus, spleen and lymph nodes were prepared and stained with the appropriate antibodies (as above). Cells were then sorted directly into Eppendorfs containing 100μl PBS, or if more than 10⁶ cells were collected they were sorted into FACS tubes again containing 100μl PBS. All sorting was carried out using the FACS Vantage (Becton Dickinson).

2.7. Molecular Biology Methods

2.7.1. Agarose gel electrophoresis

Resolution of DNA fragments for analytical or preparative purposes was by agarose gel electrophoresis, using a Gibco-BRL 'Horizon' 11.14 tank. All DNA samples were electrophoresed in standard agarose in 1x TAE buffer. Agarose (0.7 - 3%, Seakem) was melted in 1x buffer, allowed to cool, and ethidium bromide added to 0.5μg/ml. The agarose was poured into a gel casting tray with an appropriate gel comb to form loading wells. DNA samples were loaded into wells with one sixth volume of 6x loading buffer: 30% glycerol (v/v), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol (w/v). 1kb DNA ladder
(Boehringer) was also loaded as a DNA molecular weight marker. Gels were electrophoresed at 50-100 V. DNA was visualised on an ultra violet light box and photographs taken using a Polaroid land camera.

2.7.2. Transgenic mouse generation

2.7.2A. Transgene DNA purification for injection.

For details of the transgenic constructs used see the specific method sections in Chapters 4 and 5 (sections 4.2.1 and 5.2.1). The transgene was linearised using an appropriate restriction enzyme so as to give more than 8μg of insert when run on a 0.7% TAE gel. The gel was run slowly overnight until the transgene was separated from any residual vector DNA. The band of interest was excised from the gel and purified using a Wizard DNA column as previously described. The DNA was ethanol precipitated, washed with 70% ethanol and dried at 37°C until all residual alcohol had evaporated. The DNA was resuspended in 30μl of transgenic injection buffer and its concentration estimated. The DNA concentration was estimated using spectrophotometer and also by comparing on an agarose gel the intensity of the transgene band when exposed to UV light compared to some serial dilutions of a plasmid of a similar size (Range 15ng, 50ng, 100ng, 250ng, 500ng and 750 ng). The transgene was diluted to 2-4ng/μl in transgenic injection buffer and centrifuged for 15 minutes at 13000 rpm to remove any particulate matter. Leaving the residual 10-20μl, the transgene was divided into 30μl aliquots and sent off to be microinjected.

Transgenic Injection Buffer 10mM Tris, 0.1mM EDTA, ultrapure water

The buffer was filter sterilised and stored at -20°C.

2.7.2B. Microinjection of transgenic constructs

Transgenic mice were generated by Ian Rosewell (ICRF Biological Resources Unit, Clare Hall). The transgenes were purified as above and injected into pronuclei of Day 1 fertilised F1 oocytes (CBA x C57BL/6). Each construct was injected over a period of 1-2 days so that approximately 300 embryos were injected. Injected fertilised oocytes were transferred into Day 1 pseudopregnant foster mice. 20-25% of the embryos lead to a birth and generally 50-65 were
screened for integration of the transgene. The rate of integration usually fell in the range of 5-20%, giving 2-3 founder lines for each injection series. Founder mice were bred to establish stable lines and screening performed by Southern blot analysis for the first few generations to ensure integration was stable and in the one locus.

2.7.2C. Identification of Transgenic founders

Transgenic founders were identified by Southern blot hybridisation of genomic DNA extracted from tail snips and digested with EcoR1. EcoR1 cuts at the 5' and 3' ends of the cloned transgene removing a fragment of known size that can be detected by hybridisation with a transgene-specific probe comprising sequences from the human growth hormone gene. This sequence was obtained via digestion of the proximal promoter expression cassette with BamH1 and HindIII. The relative copy number of each of the lines was estimated by stripping the blots and re-hybridising with a genomic probe specific for CBFα1, an endogenous gene present at a single locus. (This Xho1-BglIII sequence was obtained as described in (Otto et al., 1997)). The relative copy number of each founder was quantitated using a PhosphorImager and accompanying ImageQuant software and was normalised to the CBFα1 signal. Founders were backcrossed to establish stable lines. Mice were bred and maintained in the ICRF Biological Resources Unit under barrier conditions.

2.7.3. Breeding

All mice were maintained in a barrier facility to ensure a Specific Pathogen Free (SPF) health status. Mice were bred at sexual maturity (6-7 weeks) and tissue taken for screening at 10-21 days.

2.7.4. Extraction of Lymphoid tissue from mice

Mice were sacrificed in a CO₂ gas chamber. Mice were dissected and the lymphoid tissues extracted. Single cell suspensions from thymus, spleen and lymph nodes were prepared by gently disaggregating the tissues on a piece of gauze with a syringe plunger in FACS buffer.
2.7.5. Fluorescence in situ hybridisation

This work was kindly performed by Jill Williamson, Human Cytogenetics laboratory. Metaphase spreads were prepared from spleens of the transgenic mice. Con A-stimulated lymphocytes were cultured at 37°C for 48 hours. The DNA probe (the human growth hormone specific sequence) was biotinylated by nick translation with biotin-14-ATP using a Bionick kit (BRL). Hybridisation to the chromosome spread was performed using standard protocols (Pinkel et al., 1986). Each biotin-labelled DNA was mixed with hybridisation solution at a final concentration of 10mg/ml and used at 100ng/slide. The hybridised probe was detected by means of Texas-red conjugated avidin (Vector Laboratories). Chromosomes were counter-stained with 4′,6-diamidino-2-phenyindole (DAPI) diluted in antifade (pH8). DAPI staining allows mouse chromosomes to be distinguished due to the reproducible banding patterns that are accentuated by these dyes. Chromosome paints (Cambio, Cambridge, UK) were used to aid in chromosome identification. Images were captured using a Zeiss Axipscope microscope equipped with a CDD camera (Photometrics). Separate images of probe signal and counterstain were merged using an Apple Powermac 8100 with Smartcapture software (Vysis, UK).
Chapter 3

The expression of p56\textsuperscript{Lck} during thymocyte development

3.1. Introduction

The work presented in this chapter investigates the expression pattern of the Src-family protein tyrosine kinase (PTK) p56\textsuperscript{Lck} during thymocyte development. The major role for p56\textsuperscript{Lck} in early thymocyte differentiation has already been introduced in Chapter 1. Gene targeting experiments generating mice that are deficient in p56\textsuperscript{Lck} have been discussed; these mice show a severe reduction in thymocyte cellularity with an incomplete arrest in thymocyte development at the CD44\textsuperscript{+}\text{CD25}\textsuperscript{+} double negative thymocyte (DN3) stage (Molina et al., 1992). Thymocyte development is more profoundly arrested in transgenic mice expressing a dominant negative p56\textsuperscript{Lck} transgene, as evidenced by the complete lack of DP cells and a total developmental arrest at the DN3 thymocyte stage (Levin et al., 1993). The role of this kinase in CD4/CD8 lineage selection and signalling from the \(\alpha\beta\)TCR complex has also been described (see sections 1.4.2.A, 1.6.1 and 1.6.2). Despite the critical role of p56\textsuperscript{Lck} in thymocyte development, little is known about the control of its expression in the thymus. The work presented here investigates the developmental timing of p56\textsuperscript{Lck} by analysing the expression of p56\textsuperscript{Lck} transcripts and protein in each of the DN thymocyte subsets.

3.1.1. An Introduction to Protein tyrosine kinases

Protein tyrosine kinases (PTKs) are implicated in the control of both cell growth and differentiation. Signal transduction in many cell types, including thymocytes, relies on the activity of PTKs. Over 24 PTKs have been identified thus far, initially as products of oncogenes. Most of these genes belong to one of two families based on their structural features. The first group are
transmembrane receptor tyrosine kinases, including growth factor receptors such as c-kit. These PTKs are characterised by having an extracellular ligand binding domain linked to a cytoplasmic catalytic domain. The second family of non-receptor PTKs is subdivided into eight groups based on sequence and function similarities (Figure 3.1 and for review see Courtneidge 1994). Of these, the Src and Syk families will be discussed due to their implicated role in thymocyte development and TCR function. Of the eight Src PTKs, four are predominantly expressed in haematopoietic cells; p56^{Lck} and p55^{Btk} are mainly restricted to lymphocytes and p59^{Hck} and p57^{c-fgr} are expressed mainly in

**Figure 3.1: Protein Tyrosine Kinases**

PTKs are divided into receptor and non-receptor tyrosine kinases, of which there are eight groups as shown below. The members of the different families are as follows: Src: Src, Fyn, Yes, Yrk, Fgr, Hck, Lyn, Lck and Blk; Csk; Fak; Jak1: Jak1, Jak2 and Tyk2; Fps: Fps and Fer; Syk: Syk and ZAP-70; Abl: Abl and Arg; Tec: Tec, Itk, Btk, Rlk, Bmx and Dsrc29.
granulocytes and monocytes. Other members are less restricted; for example, p59\textsuperscript{Fyn} is expressed in fibroblasts, platelets, lymphocytes and in neuronal tissue (for review see Cooke and Perlmutter 1989; Bolen et al., 1991).

### 3.1.2. PTK Structure

Members of the Src family share a number of common structural features (see Figure 3.2 and for review Chan et al., 1994). Firstly, to allow for membrane localisation p59\textsuperscript{Fyn} and p56\textsuperscript{Lck} both possess an N-terminal myristylated glycine at residue 2. They have a “unique domain” of approximately 80 amino acids which is thought to be important in conferring the specific associations of the kinase via interactions with other proteins. Interactions with other proteins are also mediated by the Src-homology-3 (SH3) domain, that interacts with signalling molecules with proline-rich regions, and the Src-homology-2 (SH2) domain that can interact with tyrosine phosphoproteins. At the carboxyl terminal is the kinase domain, followed by sequences including a negative regulatory tyrosine. The activity of the Src family of PTKs is regulated by protein tyrosine phosphatases (PTPases), including CD45, and by other tyrosine kinases. Phosphorylation of the negative regulatory tyrosine residue at amino acids 505 of p56\textsuperscript{Lck} and 531 of p59\textsuperscript{Fyn} inhibits catalytic activity and dephosphorylation by CD45 stimulates kinase activity. Mutation of these tyrosine residues induces constitutive kinase activity (Reynolds et al., 1987). By comparison the Syk family PTKs are not myristylated, do not contain SH3 domains or known sites of negative regulation at their carboxyl terminus and they possess two SH2 domains (Taniguchi et al., 1991).

### 3.1.3. Identification and expression of p56\textsuperscript{Lck}

p56\textsuperscript{Lck} was first discovered due to being over-expressed in a Moloney murine leukaemia virus (MoMuLV) transformed lymphoma cell line LSTRA (Marth et al., 1985; Voronova and Sefton 1986). Following from this, there are several lines of evidence to implicate p56\textsuperscript{Lck} in carcinogenesis. p56\textsuperscript{Lck} mRNA has been detected in selected cell lines derived from human T-cell leukaemias and B-cell lymphomas (Veillette et al., 1987; Sartor et al., 1989).
The p56\textsuperscript{Lck} gene is located on human chromosome 1 at position 1p32-35 near a site of frequent structural abnormalities in non-Hodgkins lymphoma and neuroblastomas (Marth et al., 1986). In addition, transfection of a p56\textsuperscript{Lck} mutant with elevated tyrosine kinase activity induced the transformation of NIH 3T3 fibroblasts (Marth et al., 1988a). Analysis of transgenic mice generated using the proximal promoter to drive expression of wild-type p56\textsuperscript{Lck} or a mutated constitutively activated p56\textsuperscript{Lck} gene showed that overexpression of p56\textsuperscript{Lck} in murine thymocytes induces thymic tumorigenesis (Abraham et al., 1991b). Overexpression of p56\textsuperscript{Lck} is also implicated in the pathogenesis of 20% of retrovirally induced lymphoid malignancies in rats (Shin and Steffen 1993). Since overexpression and/or inappropriate expression of p56\textsuperscript{Lck} can result in the development of malignancies tight control of expression of this gene is vital in normal cells.

A high level of p56\textsuperscript{Lck} transcripts has been detected in thymocytes, resting T cells and in Natural Killer cells, with a lower level in some B cell lines (Marth et al., 1985; Perlmutter et al., 1988; Biondi et al., 1991). Studies investigating signal transduction pathways in mature CD4 and CD8 T cells revealed that p56\textsuperscript{Lck} protein is expressed in these cells and that it physically associates with
the CD4 and CD8 co-receptors (Veillette et al., 1988). Similar studies also showed the presence of p56\textsuperscript{Lck} protein within immature DP cells (Veillette et al., 1989b). A more recent study of the levels of p56\textsuperscript{Lck} during thymocyte development has demonstrated a similar level of protein in the DN, DP and SP thymocyte subsets (Olszowy et al., 1995). This work did not, however, investigate the expression of p56\textsuperscript{Lck} within the individual DN thymocyte subsets. Despite being described previously as lymphocyte specific, it should be mentioned that p56\textsuperscript{Lck} protein has also been detected in mouse brain and retinal neurons (Omri et al., 1996; Omri et al., 1998). A role for p56\textsuperscript{Lck} in retinal development has been suggested following the discovery of retinal dysplasia in mice deficient for p56\textsuperscript{Lck} (Omri et al., 1998).

3.1.4. Function

As described in Chapter 1 p56\textsuperscript{Lck} plays an important role in thymocyte development and TCR mediated signalling (sections 1.4.2.A, 1.6.1 and 1.6.2). Other roles for this kinase to be mentioned here include the transduction of signals downstream from the pre-TCR and a role in allelic exclusion.

3.1.4A. Role in pre-TCR signalling

Several lines of evidence indicate a role for p56\textsuperscript{Lck} in the transmission of pre-TCR signals (see Table 3.1). Analysis of the DN compartment of mice deficient for p56\textsuperscript{Lck} revealed an increase in the percentage of cells at the DN3 thymocyte stage (Wallace et al., 1995). This could suggest that lack of p56\textsuperscript{Lck} impairs TCR\(\beta\) chain rearrangement or expression, thereby arresting cells at this stage. However, a TCR\(\beta\) chain is expressed on the cell surface of both thymocytes and T cells from the p56\textsuperscript{Lck} deficient mice and these TCR\(\beta\) chains show a normal diversity of V, D and J segment usage at the TCRV\(\beta\) locus (Penninger et al., 1996). It is therefore not surprising that the expression of a transgenic TCR\(\beta\) chain fails to restore normal thymocyte development in either 56\textsuperscript{Lck} deficient mice or in mice expressing a dominant negative p56\textsuperscript{Lck} transgene (Anderson et al., 1993b; Wallace et al., 1995). This is in contrast to the situation in recombinase-activating gene (RAG) deficient mice (which cannot rearrange their TCR\(\beta\) chain genes) where the introduction of a transgenic TCR\(\beta\) chain leads to
Table 3.1: An overview of references implicating a role for p56<sup>Lck</sup> in transduction of pre-TCR mediated signals

<table>
<thead>
<tr>
<th>Reference</th>
<th>pTα</th>
<th>Express WT TCRβ</th>
<th>RAG</th>
<th>p56&lt;sup&gt;Lck&lt;/sup&gt;</th>
<th>Tg TCR αβ/β</th>
<th>Anti-CD3</th>
<th>Dom. Neg P56&lt;sup&gt;Lck&lt;/sup&gt;</th>
<th>F505 P56&lt;sup&gt;Lck&lt;/sup&gt;</th>
<th>DN to DP transition</th>
<th>Reference</th>
</tr>
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<td>WT</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NO</td>
<td>(Mombaerts et al., 1992a)</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Partial</td>
<td>(Shinkai et al., 1992)</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Partial</td>
<td>(Shinkai et al., 1993, Shinkai and Alt 1994)</td>
</tr>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
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<td>+</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<td>(Anderson et al., 1993)</td>
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the generation of DP thymocytes (Shinkai et al., 1993). This implies a role for p56\textsuperscript{Lck} downstream of TCR\(\beta\) rearrangement and expression and provides a possible link between p56\textsuperscript{Lck} function and signalling downstream of the pre-TCR.

In addition, the introduction of a constitutively activated p56\textsuperscript{Lck} transgene, in which the negative regulatory tyrosine is mutated to a phenylalanine, into either RAG or pT\(\alpha\) deficient mice leads to the generation of more DP thymocytes (Mombaerts et al., 1994; Fehling et al., 1997). Restoration of thymocyte development by the expression of activated p56\textsuperscript{Lck} in mice that are unable either to rearrange or to express their TCR\(\beta\) genes again indicates a role for p56\textsuperscript{Lck} downstream of the pre-TCR. The central role of Src tyrosine kinases in the progression of thymocytes through the DN to DP thymocyte checkpoint is underscored in mice deficient in both p56\textsuperscript{Lck} and p59\textsuperscript{Fyn}, in which development is completely arrested at the pre-TCR-mediated checkpoint stage (van Oers et al., 1996b). Taken together, these data indicate that p56\textsuperscript{Lck} plays a major role in the transmission of pre-TCR-mediated signals that promote thymocyte survival and proliferation.

3.1.4.B. Role in allelic exclusion

The assembly and expression of a functionally rearranged TCR\(\beta\) chain as part of the pre-TCR normally results in abrogation of further rearrangements at the \(\beta\) locus. This process is known as allelic exclusion. The mechanisms used by TCR\(\beta\) chains to deliver these signals are unknown but studies have implicated the involvement of p56\textsuperscript{Lck}. Overexpression of p56\textsuperscript{Lck} inhibits TCR\(\beta\) chain rearrangement since thymocytes from transgenic mice which overexpress a constitutively active form of p56\textsuperscript{Lck} show a reduction in V\(\beta\)-D\(\beta\) rearrangement (Anderson et al., 1992). If, as outlined above, the TCR\(\beta\) chains deliver signals via p56\textsuperscript{Lck} one could predict that altering the activity of p56\textsuperscript{Lck} would disrupt the process of allelic exclusion. Thus, the introduction of a rearranged transgenic TCR may no longer have any effect. Thymocytes from mice expressing a catalytically inactive form of p56\textsuperscript{Lck} in addition to a TCR\(\beta\) transgene have defects in allelic exclusion with substantial levels of endogenous V\(\beta\) rearrangements.
despite the presence of the transgenic TCRβ (Anderson et al., 1993b). However, allelic exclusion is almost complete in p56Lck deficient thymocytes that express a TCRβ/αβ transgene, although an increased number of peripheral T cells expressing more than one Vβ chain is seen (Wallace et al., 1995). This indicates that interference with p56Lck function results in abrogated/incomplete allelic exclusion, thus implying a role for this kinase in these processes.

3.1.4. Other Roles for p56Lck

In addition to the roles for p56Lck in thymocyte development and signal transduction from the pre-TCR and αβTCR, other functions have been described for this kinase. p56Lck associates with numerous other cell surface receptors in T cells. These interactions potentially result in the activation of the PLCγ1, p21ras and PI3 kinase pathways, a number of serine-threonine kinase pathways such as the mitogen-activated protein kinase (MAPK) pathway and p72raf pathways, as well as pathways downstream of certain phosphatases (see figure 3.3 and refer to Anderson et al., 1994). Thus, through association with the pre-TCR and αβTCR complexes, as well as other cell surface receptors p56Lck plays important roles in the development and function of thymocytes and peripheral T cells.

3.1.5. Control of p56Lck gene expression

Despite the critical role of p56Lck in thymocyte development, little is known about the control of its expression in the thymus. Work investigating the structure of p56Lck mRNAs in the LSTRA and Thy19 MoMuLV thymoma cell lines identified the presence of a normal transcript and a chimeric transcript containing the 5′ untranslated region (5′UTR) of Moloney murine leukaemia virus mRNA (Voronova et al., 1987). Interestingly, in the case of LSTRA cells, MoMuLV proviral insertion resulted in a 7-fold increase in p56Lck mRNA and a 50-fold increase in protein (Marth et al., 1988b). When uninfected murine T cells were studied, these were also found to contain two forms of p56Lck transcript, differing with respect to their 5′UTRs (Voronova et al., 1987). This information led the investigators to the proposed existence of at least two promoters controlling the expression of the p56Lck gene. This speculation was confirmed by the identification of two separate promoters that generate two distinct
transcripts differing only in the sequence of 5'UTR (Adler et al., 1988). The distal promoter predominates in mature SP T cells, while the proximal promoter is active in developing thymocytes (Garvin et al., 1988; Reynolds et al., 1990; Wildin et al., 1991; Allen et al., 1992). These two promoters share little sequence homology and all the evidence suggests that they function completely independently (Takadera et al., 1989; Wildin et al., 1991). A differential usage of these two promoters has been demonstrated during T cell activation (Leung and Miyamoto 1991). Treatment of leukaemic T cell and Jurkat T cell lines with the phorbol ester tetradecanoylphorbol acetate (TPA) resulted in a downregulation of expression of transcripts from the proximal promoter and an upregulation of transcripts from the distal promoter. These different levels of p56Lck transcripts did not result from differences in RNA stability. The generation of transgenic mice expressing the SV40 large T antigen under the control of the distal or the
proximal promoter sequences has confirmed the differential patterns of expression and the independent function of these two promoters. Mice expressing this transgene under the control of the distal promoter sequences develop tumours of the lymph nodes, spleen and gross enlargement of the thymus (Wildin et al., 1991). In contrast, mice expressing this transgene under the control of the proximal promoter sequences show disrupted thymocyte differentiation and develop tumours within the thymus only (Garvin et al., 1990).

In the mouse, the proximal promoter is located directly upstream of the first p56Lck coding exon with the distal promoter being located at least 10 kilobases upstream (Adler et al., 1988). Analysis of the proximal promoter has resulted in the identification of an approximately 550 base pair fragment that can direct tissue specific and developmentally appropriate expression of the murine p56Lck gene (Allen et al., 1992). No TATA or CAAT box binding sites are present, but this region does contain numerous binding sites for transcription factors including T-cell-specific nuclear factors TCF-1 and LEF-1. Myb and Ets-related transcription factors have since been shown to be required for the activity of the proximal promoter (Leung et al., 1993; McCracken et al., 1994). In view of the above discussion regarding the link between inappropriate expression of p56Lck and malignancies the importance of this somewhat complex promoter organisation is clear.

Despite the frequent use of the p56Lck proximal promoter to express thymocyte specific transgenes (Chaffin et al., 1990; Garvin et al., 1990; Abraham et al., 1991b; Abraham et al., 1991a; Anderson et al., 1992; Levin et al., 1993) there is currently only limited knowledge about its expression in the various immature thymocyte subsets. To investigate the developmental timing of p56Lck we determined the expression of transcripts and protein in each of the DN thymocyte subsets. In addition, we generated transgenic mice expressing green fluorescent protein (GFP) under the control of the p56Lck proximal promoter (see Chapter 4). Taken together, our results show that the expression of the p56Lck gene is developmentally regulated at the post-transcriptional level at the precise
thymocyte stage at which its upstream receptor complex, the pre-TCR, is expressed.

3.2. Specific Methods

3.2.1. Details of oligonucleotides used for RT PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name of oligonucleotide</th>
<th>Sequence</th>
<th>Other Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>p56&lt;sup&gt;Lck&lt;/sup&gt;</td>
<td>Lck1</td>
<td>5'-gcccatccggaatggctctg-3'</td>
<td>Product of approximately 600 base pairs. Lck1 is from within the second exon and Lck2 is from the seventh, so the product can be distinguished from genomic DNA. Anneals at 56°C</td>
</tr>
<tr>
<td></td>
<td>Lck2</td>
<td>5'-cccaccatggtttctgggc-3'</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Actin1</td>
<td>5'-ctgacggccaggtcatca-3'</td>
<td>Product of approximately 450 base pairs. These primers also span exon-exon boundaries so the product can be distinguished from genomic DNA. Anneals at 56°C</td>
</tr>
<tr>
<td></td>
<td>Actin2</td>
<td>5'-agaccaaagccttcataacatc-3'</td>
<td></td>
</tr>
<tr>
<td>RAG-1</td>
<td>RAG1 forward</td>
<td>5'-ccaagctgcagacattctagcactc-3'</td>
<td>Product of approximately 560 base pairs, spans the only intron, does not amplify genomic DNA. Anneals at 57°C</td>
</tr>
<tr>
<td></td>
<td>RAG1 reverse</td>
<td>5'-caacatctgccttcacgtcgatcc-3'</td>
<td></td>
</tr>
<tr>
<td>CD25</td>
<td>CD25 forward</td>
<td>5'-ctctggctgtcattgacatttc-3'</td>
<td>Product of approximately 120 base pairs. Anneals at 58°C</td>
</tr>
<tr>
<td></td>
<td>CD25 reverse</td>
<td>5'-cagcaagcgtggctccatcttc-3'</td>
<td></td>
</tr>
</tbody>
</table>

3.2.2. Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

The RNA was prepared and the RT reaction was carried out as described in sections 2.3.1 and 2.3.4. The Semi-quantitative PCR reactions carried out in a 100μl volume, using the same PCR buffer conditions and dNTP concentrations as described previously. The cDNA was amplified using primers shown above (Lck1 and Lck2 or Actin1 and Actin2). 25μl of the reaction was removed after 20, 25 and 30 cycles and a 20μl portion of each PCR product was electrophoresed through a 1% agarose gel. The gel was blotted for 2 to 3 hours, and the DNA immobilised onto the membrane via UV cross linking. The blots were hybridised with a p56<sup>Lck</sup>-specific probe and with a β-actin-specific probe. These probes consisted of the sequences between the Lck1 and Lck2 primers or the Actin 1 and Actin 2 primers, respectively,

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which were amplified via PCR from cDNA from total thymocytes, cloned into the TA cloning kit (Invitrogen, according to manufacturer's protocol) and digested with EcoRI prior to labelling with $^{32}$P dCTP. These p56$^{Lck}$-specific and β-actin-specific probes were also used for hybridisation of Northern blots for the detection of p56$^{Lck}$ and β-actin transcripts, respectively. In addition, a probe specific for murine GAPDH was used to allow for differences in the amount of RNA loaded (see section 2.3.3B for details).

3.2.3. Single cell reverse-transcriptase polymerase chain reaction (SC RT-PCR)

Cells were sorted using the FACS-Vantage equipped with an automatic cell deposition unit (Becton Dickinson). Single cells were directly sorted into each well of 96-well plates (Costar, Cambridge, MA) containing 5µl PBS. Plates were stored at -70°C until use in PCR analysis (technique adapted from ten Boekel et al., 1998). cDNA was prepared with random hexamers and reverse transcribed with MMLV-Reverse transcriptase (RT), directly in the well of the 96 well plate using a thermal cycling machine (PTC-100 Programmable Thermal Controller, MJResearch Inc). First, the cells were heated to 65°C and placed on ice before addition of 10µl of pre-prepared RT reaction mixture containing RT buffer (50mM Tris HCl (pH8.3), 75mM KCl, 3mM MgCl$_2$ supplied with RT), 15mM DTT, 40U RNase block (ribonuclease inhibitor, Stratagene), 1mM final dNTPs (Ultrapure set available from Pharmacia), 1.5U random hexamers (pd(N6) Pharmacia) and 50U MMLV RT. The reactions were incubated at 37°C for 60 minutes followed by 3 minutes at 95°C to denature the RT. The plate was removed from the PCR machine and placed on ice before addition of the PCR reaction components. PCR amplification was carried out in two rounds, the first of which was again performed within the well. 75µl of pre-prepared PCR reaction mixture containing 1X PCR buffer (50mM KCl, 10mM Tris-HCl pH9 at 25°C, 0.1% Triton X-100, 1.5mM MgCl$_2$), 0.2mM dNTPs, 10pM of each oligonucleotide primer and 1U of PicoTaq polymerase was added to each well before amplification. Primers used were as above (Lck 1+2, Actin 1+2). DNA was amplified for 40 cycles of 50 seconds denaturing at 94°C, 60 seconds annealing at 56°C and 60 seconds extending at 72°C. Following amplification
the sample was transferred into a clean tube and centrifuged. 1μl of the PCR product was used in the second round of PCR reactions. The second round of PCR reactions were carried out in 20μl volume using the same primers and conditions as in the first round. DNA was amplified for 30 cycles of 20 seconds denaturing at 94°C, 30 seconds annealing at 56°C, 60 seconds extending at 72°C. A 15μl portion of each amplified product was examined by 1% agarose gel electrophoresis and stained with ethidium bromide.

3.2.4. In vitro Kinase assay

In vitro kinase assays were performed as described previously (Gilmour and Reich 1994). DN thymocytes were washed twice with PBS and lysed with 1% Triton lysis buffer (see section 2.4.1) with 1mM sodium vanadate. Lysates were precleared of non-specific antigen-antibody complexes to reduce background with 20μl protein A-Sepharose for 30 minutes at 4°C. Following centrifugation for 5 minutes at 13000 rpm at 4°C, the supernatant was transferred into a clean Eppendorf and immunoprecipitations were performed. A 100-fold-dilution of rabbit anti-human p56Lck polyclonal IgG was added and the lysate/antibody mixture was mixed overnight by rotation at 4°C. Immune complexes were captured via addition of 30μl of anti-mouse agarose beads, a further hour rotation at 4°C and centrifugation for 5 minutes at 13000rpm at 4°C. Immune complexes were washed twice with 1mM sodium vanadate in PBS and once with kinase buffer before resuspension in kinase buffer containing 20μCi of [α-32P] ATP (>4000 Ci/mmol; ICN Pharmaceuticals, CA, USA). Following incubation at room temperature for 5 minutes the immune complexes were washed once with kinase buffer. The samples were analysed by SDS-PAGE on 12% polyacrylamide gels and transferred electrophoretically onto Immobilon-P poly(vinylidene difluoride) membranes (Millipore).

Kinase buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM HEPES (pH 7.5)</td>
<td>5mM MnCl₂</td>
</tr>
<tr>
<td>100mM NaCl</td>
<td>5mM MgCl₂</td>
</tr>
</tbody>
</table>

3.2.5. Intracellular staining for p56Lck

The population of interest was sorted and washed twice in PBS before being fixed in 1% paraformaldehyde for 20 minutes at room temperature. The
cells were washed twice in PBS before being resuspended in 0.3% saponin buffer (SB), (0.3% saponin (SIGMA, St. Louis, MO), 10mM Hepes, 2% FCS, in PBS) and incubated for 10 minutes at room temperature. The cells were washed once in 0.3% SB before staining in 0.1% SB for 30 minutes at room temperature with a rabbit polyclonal anti-p56<sup>Lck</sup> antibody (gift from Dr S Ley) or a control rabbit pre-immune serum at the same concentration. The cells were washed twice in 0.1% SB before incubation for a further 30 minutes at room temperature with FITC-conjugated swine anti-rabbit immunoglobulin (DAKO Ltd, Cambridge, UK). Cells were washed and analysed on the flow cytometer as described in Chapter 2.

3.3. Results

3.3.1. Expression of p56<sup>Lck</sup> transcripts within the early thymocyte subsets

To analyse the expression of p56<sup>Lck</sup> mRNA, as well as other genes expressed within the early thymocyte populations, reverse-transcriptase polymerase chain reaction (RT-PCR) was carried out. Cells from the four double negative (DN) thymocyte subsets (DN1; CD44<sup>+</sup>CD25<sup>-</sup>, DN2; CD44<sup>+</sup>CD25<sup>+</sup>, DN3; CD44<sup>+</sup>CD25<sup>+</sup>, and DN4; CD44<sup>-</sup>CD25<sup>-</sup> cells) were sorted and total RNA was extracted (see section 2.6.2 for details of the antibodies used for the preparation of the DN subsets). In addition, RNA was obtained from total thymocytes and splenocytes from C57BL/6 mice and from thymocytes from mice deficient for p56<sup>Lck</sup>. Following cDNA synthesis, primers specific for β-actin, p56<sup>Lck</sup>, RAG-1 and CD25 were used in the PCR, consisting of 30 cycles. As shown in Figure 3.4, products specific for p56<sup>Lck</sup> were obtained in all four DN thymocyte subsets, as well as in total thymocyte and splenocyte samples from WT mice. No product was obtained for the cDNA derived from p56<sup>Lck</sup> deficient thymocytes demonstrating the specificity of the primers used. All of the samples tested were shown to express CD25 transcripts. Rag-1 was detected in the thymus and in the p56<sup>Lck</sup> deficient thymocytes but was not expressed in the splenocyte sample. Within the DN subsets Rag-1 were detected in the DN2, DN3 and DN4 samples but absent in the DN1 population.
Figure 3.4: RT-PCR analysis of the expression of p56\textsuperscript{Lck}, CD25 and RAG-1 during thymocyte development

RNA was made from 5 x 10\textsuperscript{4} cells from various thymocyte subsets from C57BL/6 and cDNA was prepared. PCR was carried out with specific primers shown. Abbreviations used: DN 1, CD44\textsuperscript{+}CD25\textsuperscript{+} cells; DN2, CD44\textsuperscript{+}CD25\textsuperscript{+} cells; DN3, CD44 CD25\textsuperscript{+} cells; DN4, CD44 CD25\textsuperscript{+} cells; T, thymocytes; S, splenocytes and p56\textsuperscript{Lck}\textsuperscript{-/-} T, thymocytes from p56\textsuperscript{Lck}\textsuperscript{-/-} deficient mice.
The above preliminary analysis suggests that p56\textsuperscript{Lck} transcripts are expressed from the DN1 population and are present throughout early thymocyte development. This RT-PCR does not allow for quantitation of the levels of p56\textsuperscript{Lck} present. For this reason the expression of p56\textsuperscript{Lck} transcripts was further analysed by semi-quantitative RT-PCR and ultimately by Northern blotting. Semi-quantitative RT-PCR allows for the relative level of expression of genes between samples to be estimated. Cells from the four DN thymocyte subsets, DP, SP cells and macrophages were sorted and total RNA was isolated. Macrophages are deficient for p56\textsuperscript{Lck} expression and so act as a control for these reactions. Following cDNA synthesis PCR was performed using specific primers for p56\textsuperscript{Lck} and β-actin (as above). Products specific for p56\textsuperscript{Lck} were obtained in all four DN thymocyte subsets as well as in the DP and SP samples (see Figure 3.5). In the DN1 sample a specific p56\textsuperscript{Lck} product was detectable after 25 cycles, whereas from the DN2 sample onwards a specific product was detectable after 20 cycles. This indicates p56\textsuperscript{Lck} is expressed at a similar level within the DN2, DN3, DN4, DP and SP thymocyte samples and at a lower level within the DN1 thymocyte sample. The macrophage samples did not express p56\textsuperscript{Lck} transcripts.

To confirm the above results and assess the steady state levels of p56\textsuperscript{Lck} expression within thymocyte development Northern blot analysis was carried out. RNA was extracted from cells from the four DN thymocyte subsets, as well as from total thymocytes and from heart. As shown in Figure 3.6, p56\textsuperscript{Lck} transcripts were clearly detectable in each DN thymocyte subset and in the total thymocytes but were absent from heart. When normalised to the level of glyceraldehyde 3-phosphate dehydrogenase mRNA, there was a slight upregulation of p56\textsuperscript{Lck} transcripts between the first and second DN subsets, but this was less than two-fold. These results demonstrate that the p56\textsuperscript{Lck} gene is transcribed at the earliest identifiable stage of thymocyte development. p56\textsuperscript{Lck} mRNA is expressed throughout thymocyte development at relatively uniform levels.
Figure 3.5: Semi quantitative RT-PCR analysis of p56\textsuperscript{Lck} expression in sorted thymocyte subsets

RNA was made from 5 x 10\textsuperscript{4} cells from various thymocyte subsets from which cDNA was prepared. PCR reactions were carried out as described in Chapter 2, with 25\mu l of the reaction being removed after 20, 25 and 30 cycles. A 20\mu l portion of each amplified product was then electrophoresed through a 1\% agarose gel. The gel was then blotted and the DNA immobilised onto the membrane via UV cross linking. The blots were hybridised with an p56\textsuperscript{Lck} -specific probe before being stripped and re-hybridised with a \beta-actin-specific probe. Abbreviations used: DW, distilled water; 1, DN1 cells; 2, DN2 cells; 3, DN3 cells; 4, DN4 cells; DP, double positive cells; SP, single positive cells; M, macrophages.

\begin{center}
\begin{tabular}{cccccccc}

|   | DW | 1 | 2 | 3 | 4 | DP | SP | M |
\hline
p56\textsuperscript{Lck} & & & & & & & & \\
\hline
\beta-Actin & & & & & & & & \\
\end{tabular}
\end{center}
Figure 3.6: Northern blot analysis of the steady state level of p56<sup>Lck</sup> mRNA in DN thymocyte subsets

Thymocytes from C57BL/6 mice were isolated by complement dependent killing and FACS. Total RNA was prepared from 3 x 10<sup>5</sup> cells from each DN thymocyte subset and separated on an agarose gel containing formaldehyde. The blots were hybridised with the indicated probes. RNA from 10<sup>6</sup> T cells and from heart tissue were used as positive and negative controls, respectively. The intensity of the p56<sup>Lck</sup> band was quantitated using a phosphorimager and the values normalised against the GAPDH loading control. Abbreviations used: DN population 1, CD44<sup>+</sup>CD25<sup>+</sup> cells; 2, CD44<sup>+</sup>CD25<sup>+</sup> cells; 3, CD44<sup>-</sup>CD25<sup>+</sup> cells; 4, CD44<sup>-</sup>CD25<sup>-</sup> cells.

<table>
<thead>
<tr>
<th>DN population</th>
<th>T cells</th>
<th>Heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  2  3  4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Relative quantitation for p56<sup>Lck</sup>**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>1.3</th>
<th>1.5</th>
<th>1</th>
</tr>
</thead>
</table>

**Probes:**
- p56<sup>Lck</sup> (2.2 Kb)
- GAPDH (1.4 Kb)
3.3.2. Detection of $p56^{Lck}$ transcripts at a single cell level

The DN thymocyte subsets, as defined by the combinatorial expression of CD44 and CD25, comprise heterogeneous populations of cells (Penit et al., 1995; Hoffman et al., 1996). In order to determine whether this heterogeneity was reflected by heterogeneity of $p56^{Lck}$ transcript expression within the four DN thymocyte subsets, single cell RT-PCR was carried out. To confirm the specificity of the primers being used and to verify the accuracy of the technique, single macrophages and DP thymocytes were sorted into individual wells of 96 well plates. Each well containing either a macrophage or a DP thymocyte resulted in a $\beta$-actin product upon PCR (Figure 3.7A). Single DP thymocytes also resulted in specific product when subjected to PCR with the $p56^{Lck}$ primers. No $p56^{Lck}$ product was obtained with for single macrophages. Single cells from the DN1, DN2, DN3 and DN4 populations were sorted into individual wells of four 96 well plates and the RT-PCR was repeated. A representative experiment for DN1 cells is shown in Figure 3.7B. At the single cell level greater than 95% of all DN1, DN2, DN3 and DN4 cells examined expressed $p56^{Lck}$ mRNA. No PCR product was obtained for $p56^{Lck}$ or $\beta$-actin when no cell was collected.

3.3.3. Analysis of the expression of $p56^{Lck}$ protein in developing thymocytes and other leukocyte populations by intracellular staining and flow cytometric analysis

The presence of equivalent steady state levels of $p56^{Lck}$ mRNA in all four DN thymocyte subsets suggests that $p56^{Lck}$ protein is also expressed from the earliest stage of thymocyte development. In order to test this prediction, the developmental timing of $p56^{Lck}$ protein expression within the thymus and other leukocyte populations was determined. Initially the expression of $p56^{Lck}$ protein in a number of leukocyte populations was assessed. Cells from the thymus, spleen or lymph nodes of C57BL/6 mice were stained intracellularly with a rabbit polyclonal anti-$p56^{Lck}$ antibody. As shown in Figure 3.8A, over 90% of thymocytes, approximately 60% of peripheral lymph node cells and about 30%
Figure 3.7: Single cell RT-PCR analysis of p56\textsubscript{Lck} transcript expression in CD44\textsuperscript{+}CD25\textsuperscript{-}sorted cells

Single cells were sorted and subjected to reverse transcriptase polymerase chain reaction (RT-PCR) with oligonucleotide primers specific for p56\textsubscript{Lck} or \beta-actin. The results shown are representative of reactions from 4 complete 96 well plate experiments.

Figure 3.7A

<table>
<thead>
<tr>
<th>Macrophage</th>
<th>DP thymocyte</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1 1 1 1</td>
<td>1 1 1 1 1</td>
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</tbody>
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\beta-Actin

<table>
<thead>
<tr>
<th>Macrophage</th>
<th>DP thymocyte</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
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p56\textsubscript{Lck}

Figure 3.7B

<table>
<thead>
<tr>
<th>CD44+CD25- DN1 thymocyte</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1 1 1 1 0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

\beta-Actin

<table>
<thead>
<tr>
<th>CD44+CD25- DN1 thymocyte</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1 1 1 1 0 0 0 0 0 0</td>
<td></td>
</tr>
</tbody>
</table>

p56\textsubscript{Lck}
thymocytes, approximately 60% of peripheral lymph node cells and about 30% of splenocytes expressed p56\textsuperscript{Lck} protein. The cells of the lymph node and spleen that expressed p56\textsuperscript{Lck} were shown to be T cells by staining with an anti-Thy-1 antibody (Figure 3.8B). Less than 1% of B cells and macrophages expressed p56\textsuperscript{Lck} protein. Approximately 50% of natural killer (NK) cells (positive for the cell surface marker NK1.1 and CD3) and 70% of γδT cells contained p56\textsuperscript{Lck} protein. The specificity of the antibody used in these studies was demonstrated by the lack of staining of thymocytes from p56\textsuperscript{Lck}-deficient mice (Molina et al., 1992).

Thymocyte subsets were then stained intracellularly with a polyclonal p56\textsuperscript{Lck}-specific antibody (Figure 3.9). Within the DN thymocyte compartment, p56\textsuperscript{Lck} protein was not detected in the DN1 and DN2 subsets. In contrast, over 90% of the DN3 and DN4 populations expressed p56\textsuperscript{Lck} protein. Over 90% of DP and mature SP thymocytes were also positive for p56\textsuperscript{Lck} expression. When the relative p56\textsuperscript{Lck} fluorescence intensity compared to control serum was analysed for each thymocyte subset, the DN3 thymocyte subset was shown to have at least two times more p56\textsuperscript{Lck} protein as detected by intracellular staining than any other subsequent thymocyte population (data not shown).

3.3.4. Immunoblot analysis of the expression of p56\textsuperscript{Lck} protein in developing thymocytes

The expression of p56\textsuperscript{Lck} within the DN compartment was also examined by immunoblot analysis. Because of the limiting numbers of the DN subsets, two pools, each of 10\textsuperscript{5} cells were made of sorted CD44\textsuperscript{+}CD25\textsuperscript{−} plus CD44\textsuperscript{+}CD25\textsuperscript{+} cells (DN1+2) or CD44\textsuperscript{−}CD25\textsuperscript{+} plus CD44\textsuperscript{−}CD25\textsuperscript{−} cells (DN3+4). Consistent with the flow cytometry analysis described above, p56\textsuperscript{Lck} protein was barely detectable in the DN1+2 pooled cells, but was clearly expressed in the DN3+4 subsets (Figure 3.10). Densitometric analysis on a phosphorimager of an over-exposure of this blot revealed at least a twenty-fold higher amount of p56\textsuperscript{Lck} protein in the pooled DN3+4 lysate in comparison to the DN1+2 lysate.
Figure 3.8: Flow cytometric analysis of leukocyte populations stained intracellularly for p56\textsuperscript{Lck} protein

Figure 3.8A: Thymocytes, splenocytes and lymph node (LN) cells from C57BL/6 mice (4 - 6 weeks of age) were isolated, stained with monoclonal antibodies to cell surface markers, sorted and stained intracellularly with an anti-p56\textsuperscript{Lck} antibody (green lines) or with a control serum (purple lines). Thymocytes were isolated from mice deficient for p56\textsuperscript{Lck} as a control for antibody specificity. The percentage of cells falling within the marker is given. The results are representative of four different experiments.

Figure 3.8B: Splenocytes and lymph node cells from the above experiment were stained with an anti-Thy1.1 antibody
Figure 3.9: Intracellular staining of thymocyte subsets for p56\textsuperscript{Lck} protein

Thymocytes from C57BL/6 mice (4 - 6 weeks of age) were isolated, stained with monoclonal antibodies to cell surface markers, sorted and stained intracellularly with an anti-p56\textsuperscript{Lck} antibody (green lines) or with a control serum (purple lines). The percentage of cells falling within the marker is given. The results are representative of four different experiments. Abbreviations used: DN1, CD44\textsuperscript{+}CD25\textsuperscript{-} cells; DN2, CD44\textsuperscript{+}CD25\textsuperscript{+} cells; DN3, CD44\textsuperscript{-}CD25\textsuperscript{+} cells; DN4, CD44\textsuperscript{-}CD25\textsuperscript{-} cells; DP, double positive cells; CD4 SP, CD4 single positive cells; CD8 SP, CD8 single positive cells.
Figure 3.10: p56Lck Immunoblot analysis in pooled DN1 and 2 and DN3 and 4 thymocyte subsets

CD44+CD25- and CD44+CD25+ thymocytes (DN 1 +2) from C57BL/6 mice (4 - 6 weeks of age) were isolated, sorted and pooled (10^5 total) as were the same number of CD44+CD25+ and CD44+CD25- cells (DN 3 + 4). Lysates were electrophoresed, transferred to a membrane and immunoblotted with an anti-p56Lck antibody. A Jurkat cell lysate was included as a positive control and an A431 fibroblast cell lysate as a negative control. “M” indicates protein size markers. The panel below shows an overexposure of the same blot.
These data clearly demonstrate that there is a dramatic upregulation of p56\textsuperscript{Lck} protein expression within the DN thymocyte subsets as thymocytes progress from the DN2 to the DN3 stage.

3.3.5. \textit{In vitro} kinase assay analysis of the activity of p56\textsuperscript{Lck} within the DN thymocyte subsets

The absence of detectable p56\textsuperscript{Lck} protein within the DN1 and DN2 thymocytes subsets indicates that these cells lack p56\textsuperscript{Lck} kinase activity. To investigate this two pools of DN1+2 and DN3+4 thymocytes (3 x 10\textsuperscript{5} in total) were sorted. Lysates from these cells, as well as from 10\textsuperscript{6} thymocytes from C57BL/6 and p56\textsuperscript{Lck} deficient mice, were subjected to an \textit{in vitro} kinase assay. As shown in Figure 3.11, p56\textsuperscript{Lck} kinase activity was detectable in thymocytes from control C57BL/6 mice but was absent from p56\textsuperscript{Lck} deficient thymocytes. p56\textsuperscript{Lck} kinase activity was not detectable in pooled DN1+2 thymocytes, whereas activity was clearly evident in pooled DN3+4 cells.

3.3.6. Analysis of the expression of p56\textsuperscript{Lck} protein in developing thymocytes following growth in culture

The lack of expression of p56\textsuperscript{Lck} protein in DN1 and DN2 thymocytes may be because these subsets differentiate rapidly to DN3 and DN4 cells before p56\textsuperscript{Lck} protein levels can accumulate to detectable levels. To rule out this possibility, pooled DN1+2, pooled DN3+4 thymocytes and total thymocytes from C57BL/6 mice were incubated overnight in culture medium containing stem cell factor, IL-6 and IL-7, 10\% FCS and 5\% WEHI-3B conditioned medium (a source of IL-3 and multi-colony stimulating factor) (see Robinson and Riches 1991). Under these conditions cells remain viable and proliferate extensively, but fail to differentiate, presumably due to the lack of a thymic stoma. When cells were analysed by FACS for intracellular p56\textsuperscript{Lck} expression, the total thymocytes and pooled DN3+4 subsets contained detectable p56\textsuperscript{Lck} protein after 0, 24 and 48 hours in culture (Figure 3.12). In contrast, the pooled DN1+2 subsets contained no detectable p56\textsuperscript{Lck} protein even after growth for 48 hours in culture.
CD44+CD25− and CD44+CD25+ thymocytes (DN 1 +2) from C57BL/6 mice (4 - 6 weeks of age) were isolated, sorted and pooled (3 x 10^5 total) as were the same number of CD44−CD25+ and CD44−CD25− cells (DN3+4). Lysates from these cells, as well as 10^6 thymocytes from C57BL/6 mice and p56Lck deficient mice were subjected to an in vitro kinase assay. Radiolabelled immunoprecipitated proteins were separated by 12% SDS-PAGE and visualised by autoradiography.
Figure 3.12: Flow cytometric analysis of thymocyte subsets stained intracellularly for p56\textsuperscript{Lck} protein following growth in culture

DN1+2, DN3+4 and total thymocytes from C57BL/6 mice were isolated, sorted and pooled. Following incubation for 0, 24 or 48 hours in culture with IL-6, IL-7, 10% FCS and 5% WEHI-conditioned medium the cells were stained intracellularly with an anti-p56\textsuperscript{Lck} antibody (green lines) or with a control serum (purple lines). The results are representative of four different experiments.
The level of p56Lck mRNA within the thymocytes after 48 hours in culture was similar in all three groups of cells (DN1+2, DN3+4 and total thymocytes) (data not shown). Thus, despite the presence of p56Lck mRNA in DN1+2 thymocytes even after 48 hours in culture these cells fail to express p56Lck protein. Therefore, the lack of p56Lck protein in the two most immature DN thymocyte subsets was unlikely to be due to the failure to accumulate sufficient steady state levels prior to their developmental progression to more mature subsets.

3.4. Discussion

3.4.1. The role of p56Lck as a transducer of pre-TCR mediated signalling

As previously introduced, the DN to DP thymocyte transition is the first major checkpoint in thymocyte development. At this stage thymocytes that have successfully generated in-frame TCRβ rearrangements are positively selected. D to Jβ rearrangement is detected at the CD44+CD25+ (DN2) stage of thymocyte development and V to DJβ rearrangement at the CD44−CD25+ (DN3) stage (Godfrey et al., 1994; Wilson and MacDonald 1995). Successful TCRβ gene rearrangement results in the surface expression of the pre-TCR that can be detected by flow cytometry on DN3 cells but not on the DN2 population (Bruno et al., 1999). Mice that are unable to rearrange or express their TCRβ genes are blocked in thymocyte development at the DN3 stage (Mombaerts et al., 1992a; Mombaerts et al., 1992b; Shinkai et al., 1992; Fehling et al., 1995). Thus, surface expression of the pre-TCR and signalling downstream from this receptor are required for thymocyte development. Previous studies have clearly established a major role for p56Lck in the signal transduction pathway downstream of the pre-TCR (see section 3.1.4A and Table 3.1). The importance of p56Lck in thymocyte development is clear since interference with p56Lck function, via gene targeting or expression of a dominant negative p56Lck transgene, results in a block in thymocyte maturation at the DN3 stage (Molina et al., 1992; Levin et al., 1993). Unlike mice deficient for RAG, pTa or TCRβ, the introduction of a functional TCRβ transgene or addition of anti-CD3 antibodies does not result in restoration of thymocyte development in the p56Lck deficient or dominant
negative p56\textsuperscript{Lck} transgenic mice. Expression of an activated form of p56\textsuperscript{Lck} in mice deficient for RAG, p\textalpha or TCR\beta rescues thymocyte development. Therefore, a role for p56\textsuperscript{Lck} downstream of TCR\beta rearrangement and expression, as a transducer of pre-TCR mediated signalling is established.

3.4.2. The expression of p56\textsuperscript{Lck} protein is upregulated at the same stage at which its upstream receptor complex, the pre-TCR, is expressed

Previous work has demonstrated that the level of p56\textsuperscript{Lck} protein is relatively constant throughout thymocyte development, since DN, DP and SP cells all express a similar level of protein (Olszowy et al., 1995). However until now, the expression of p56\textsuperscript{Lck} within the individual DN thymocyte subsets has not been documented. The results presented here show that the steady state level of p56\textsuperscript{Lck} protein is dramatically upregulated between DN2 and DN3 thymocytes, the same stage at which the pre-TCR begins to be expressed (Figures 3.9 and 3.10). In addition, \textit{in vitro} kinase assays have revealed that p56\textsuperscript{Lck} activity is not detectable in thymocytes prior to the DN3 stage (Figure 3.11). These observations are consistent with the notion that an early role of p56\textsuperscript{Lck} in developing thymocytes is to transduce pre-TCR generated signals.

Interestingly, the median p56\textsuperscript{Lck} fluorescence intensity in DN3 thymocytes was greater than in all other thymocyte subsets analysed, even though the levels of mRNA within these cells is similar to the other thymocyte subsets (Figures 3.5 and 3.6). The difference in p56\textsuperscript{Lck} median fluorescence intensity between DN3 thymocytes and DP or SP thymocytes may be explained by differences in cell volume, since the DN3 population comprises a higher proportion of large blast-like cells. This implies that these larger cells are able to make more p56\textsuperscript{Lck} protein from the same amount of p56\textsuperscript{Lck} mRNA i.e. they have more efficient translation due to, for example, enhanced access to ribosomes or increased levels of initiation factors. This suggests that these steps are rate limiting in the smaller more mature cells. This seems unlikely given that the uniformly large DN4 thymocytes, which have a similar level of p56\textsuperscript{Lck} mRNA expression as DN3 cells, show a decreased level of protein expression. This
implies that $p56^{Lck}$ protein is expressed at a maximal level within the DN3 subset. The particularly high level of $p56^{Lck}$ protein in DN3 cells may compensate for the lack of protein in DN1 and DN2 cells and so may account for the overall similar level in the DN, DP and SP thymocytes described (Olszowy et al., 1995). Therefore, the expression of $p56^{Lck}$ protein is upregulated within the DN3 subset, at the precise stage at which its upstream receptor complex, the pre-TCR, is first expressed.

3.4.3. Despite expression of $p56^{Lck}$ transcripts within DN1 thymocytes $p56^{Lck}$ protein is not detectable until the DN3 subset

The observed upregulation in $p56^{Lck}$ protein levels in DN3 cells is not reflected at the RNA level. The steady state level of $p56^{Lck}$ mRNA varies by less than two-fold throughout development of the DN thymocyte subsets (Figure 3.6). The late expression of $p56^{Lck}$ protein therefore seems surprising in view of the fact that mRNA is detectable at the earliest identifiable stage of thymocyte development and is expressed throughout thymocyte development. Alterations in the expression of $p56^{Lck}$ have been implicated in causing neoplastic transformation in a Moloney murine leukaemia virus (MoMuLV) transformed lymphoma cell line (Marth et al., 1985). Also, transgenic mice that express a constitutively activated form of $p56^{Lck}$ develop thymic tumours (Abraham et al., 1991b). This sensitivity of thymocytes to $p56^{Lck}$-induced transformation following alterations in $p56^{Lck}$ expression underscores the importance of the tight regulation of expression of this protein.

The discrepancy between $p56^{Lck}$ expression at the mRNA and protein level may be due to the transient nature of the DN populations. This result may simply reflect the time needed for translation to occur, meaning that transcripts are required from as early as the DN1 subset to ensure the protein is available when needed in the DN3 cells. Other genes expressed in the thymus have been described as having a similar expression pattern, so possibly adding weight to this idea. For example, CD3ε mRNA has been detected by single cell in situ hybridisation in a large fraction of DN1 and DN2 cells and yet CD3ε protein is not detectable until the DN3 subset (Wilson et al., 1999). However, the protein
products of other genes that are required for the first time in the DN3 cells, such as RAG-1, as shown here (Figure 3.4) are not transcribed until the DN2 subset. The theory that p56<sup>Lck</sup> mRNA is needed as early as DN1 thymocytes, due to time constraints, is also undermined by experiments presented here. Even after 48 hours growth in culture, despite the presence of p56<sup>Lck</sup> mRNA, pooled DN1 and DN2 thymocytes contained no detectable p56<sup>Lck</sup> protein unlike their more mature counterparts (Figure 3.12). The lack of p56<sup>Lck</sup> protein in the two most immature DN thymocyte subsets is, therefore, unlikely to be due to insufficient time for translation. This implies that there is some mechanism that inhibits translation of p56<sup>Lck</sup> mRNA in DN1 and DN2 cells but not in DN3 and DN4 cells.

It is possible that p56<sup>Lck</sup> can only be detected by the antibody used in these studies (rabbit polyclonal anti-p56<sup>Lck</sup> antibody, gift from Dr S Ley) when complexed to other proteins first expressed at the DN3 stage, such as components of the pre-TCR. This seems unlikely since western blotting has confirmed that protein is undetectable in DN1 and DN2 thymocytes but is readily detectable in DN3 and DN4 cells. Due to the denaturing conditions used in this technique any complexes formed within the DN3 and DN4 cells that may not be formed in the DN1 and DN2 cells would be destroyed. In addition, p56<sup>Lck</sup> protein has been detected, via immunoblot analysis with this antibody, in RAG-1 deficient thymocytes (data not shown). Since these thymocytes do not express a functional pre-TCR this explanation seems unlikely. Alternatively, the structural conformation of p56<sup>Lck</sup> protein may be different in the DN1 and DN2 populations than in more mature subsets. It is possible that the antibody used is only able to recognise p56<sup>Lck</sup> protein as present in the later subsets due to conformational changes.

Therefore, despite the presence of p56<sup>Lck</sup> transcripts there appears to be some mechanism to control the translation of p56<sup>Lck</sup> protein in DN 1 and DN2 cells. This suggests that expression of p56<sup>Lck</sup> may be developmentally regulated at the post-transcriptional level. The mechanism of this post-transcriptional regulation of p56<sup>Lck</sup> expression in immature thymocytes is unknown, but may be
at the level of protein stability, translational regulation, or a combination of both.

3.4.4. Possible explanations and mechanisms for the post-transcriptional regulation of p56\textsubscript{Lck} expression in early thymocyte development

The discrepancy between the expression of p56\textsubscript{Lck} transcript and protein could reflect an intrinsic instability of p56\textsubscript{Lck} protein in more immature thymocytes. It is possible that an additional protein is required for stabilisation of p56\textsubscript{Lck} that is present in the DN3 and DN4 subsets but absent in DN1 and DN2 cells. For example, other components of the pre-TCR may be required to stabilise p56\textsubscript{Lck} protein. This idea is based on previous reports investigating the stability of TCR proteins in immature thymocytes (Kearse et al., 1995). This work demonstrated that TCR\textsubscript{a} proteins are inherently unstable in DP thymocytes unless associated with TCR\textsubscript{\beta} proteins. Components of the pre-TCR first expressed at the DN3 stage may be required for stabilisation of p56\textsubscript{Lck} protein. However, as mentioned, p56\textsubscript{Lck} protein has been detected in RAG-1 deficient thymocytes that do not express a functional pre-TCR, so undermining this idea. Further work investigating the differential expression of genes in DN1 and DN2 cells in comparison to DN3 and DN4 thymocytes may allow putative protein candidates responsible for this stabilisation in later DN subsets to be identified.

Alternatively, the post-transcriptional regulation of p56\textsubscript{Lck} expression in immature thymocytes may be at the level of translational regulation. Translational control is characterised by the differential utilisation of pre-existing mRNAs that can allow for rapid, efficient production of gene products without requiring RNA synthesis and processing. A frequently targeted step for translational control is translation initiation. For translation to be initiated the ribosome, consisting of a small (40S) subunit and a large (80S) subunit, must first bind to the mRNA. This requires the participation of several eukaryotic initiation factors (eIFs) as well as the m7GpppN cap structure (where N can be any nucleotide) at the 5' end and the poly-A tail at the 3' end of the mRNA. The ribosomes then "scan" the 5' untranslated region (5'UTR) until they encounter
the appropriate AUG initiator as defined by its flanking consensus sequence (for review see Kozak 1999). The presence of additional out of frame AUG codons within the 5' untranslated region (5'UTR) of an mRNA can dramatically inhibit translation of the downstream cistron, a phenomenon known as translational masking. Eukaryotic ribosomes have a low capacity to reinitiate translation so recognition of AUGs upstream of the *bona fide* AUG initiator can result in inhibition of translation of the transcript. In a survey of 699 vertebrate mRNA sequences less than 10% contained upstream AUG codons, and the subset that did mainly included oncogenes and growth factor genes (Kozak 1987).

Previous work has established a prominent role for translational control in the regulation of gene expression during T cell activation (Garcia-Sanz et al., 1998). Interference with normal translational control has also been linked with the development of certain diseases. For example, aberrant translational control of the *c-myc* gene has been implicated in the pathogenesis of multiple melanoma (Paulin et al., 1996). In this context, Perlmutter and colleagues have previously demonstrated that p56Lck expression is subject to translational regulation in a Moloney murine leukaemia virus (MoMuLV) transformed lymphoma cell line, LSTRA (Marth et al., 1988b; Perlmutter 1990). This work reported that MoMuLV proviral insertion resulted in overexpression of p56Lck transcripts that differed from normal transcripts in the sequence of the 5'UTR. The 5'UTR of normal p56Lck transcripts contain a number of out of frame 5' AUG triplets, at least one of which is in good sequence context according to Kozak's criteria, upstream of the *bona fide* AUG initiator. Proviral insertion resulted in the normal 5'UTR being replaced by non AUG-containing proviral sequences. Mutational analysis of the 5'UTR region of the p56Lck transcripts revealed that removal of these AUG codons resulted in a seven to ten-fold increase in translation of a reporter gene in comparison to the wild-type construct (Marth et al., 1988b; Perlmutter 1990). Therefore, p56Lck transcripts normally contain a 5'UTR that reduces the efficiency of translation due to translational masking. It is possible that this mechanism also operates physiologically in thymocytes. The process that could allow for recognition of the correct AUG codon within DN3 and DN4 thymocytes but not in DN1 and DN2 cells is unknown. As described by
Perlmutter, 5' AUG sequences are common within the Src family gene transcripts, for example the human p59Hck mRNA includes 6 5'AUGs (Perlmutter 1990). Expression analysis revealed that constructs containing this 5'UTR region failed to result in detectable expression of p59Hck within fibroblasts. However, p59Hck protein is clearly expressed in granulocytes, so demonstrating that cell specific mechanisms must exist to permit selective recognition of the correct AUG triplet (Perlmutter 1990).

Control of p56Lck expression in this manner may allow for the rapid expression of p56Lck protein within DN3 and DN4 thymocytes, without the need for de novo transcription of the gene. Expression of p56Lck protein would therefore be co-ordinated to coincide with TCRβ gene rearrangement and expression of the pre-TCR, enabling this cytoplasmic signalling molecule to function downstream of the pre-TCR receptor. In view of the above discussion with regards to the link between inappropriate expression of p56Lck and malignancies, the importance the tight regulation of expression of this protein is clear.

Before concluding this discussion of putative translational control of p56Lck protein expression it should be mentioned that in addition to the above mentioned role for the 5'UTR in control of p56Lck expression the 3'UTR has also been implicated. T cell activation has been shown to result in rapid decline in the levels of p56Lck mRNA and protein (Marth et al., 1987). The reduction in the steady-state levels of p56Lck mRNA was due to a decreased level of transcription and a decreased stability of transcripts (Paillard and Vaquero 1991). Studies with constructs coding for mutated p56Lck mRNAs have since shown that the 3'UTR is involved in the regulation of messenger stability (Vanhee-Brossollet and Vaquero 1997). This is only of limited relevance here, since the similar steady state levels of p56Lck mRNA within the four DN thymocyte subsets implies regulation of expression here is not the level of message stability. Previous work has therefore shown that p56Lck expression is controlled by transcriptional and post-transcriptional mechanisms, including alterations in message stability. It is
not unreasonable to suggest that $p56^{Lck}$ expression during thymocyte development may be regulated in part at the level of mRNA translation.

3.5 Future work

3.5.1. Translational masking

As described, due to the presence of additional AUGs upstream of the *bona fide* AUG initiator in the 5'UTR of the $p56^{Lck}$ transcript, one possible mechanism for translational control of $p56^{Lck}$ expression in early thymocyte development is that of translational masking. Previous work by Perlmutter and colleagues has demonstrated the inhibitory effect of the upstream AUGs on translation of $p56^{Lck}$ mRNA (Marth et al., 1988b; Perlmutter 1990). These studies involved transient transfection of NIH 3T3 fibroblasts with a number of metallothioein-1 (MT-1) promoter driven constructs with mutated or truncated 5'UTRs upstream of the $p56^{Lck}$ exon 1B. Future work aims to establish if translational masking is active in the control of $p56^{Lck}$ expression within thymocyte development. This work will utilise constructs with mutated 5'UTRs, similar to those used by Perlmutter, upstream of the full cDNA sequence for $p56^{Lck}$, followed by an internal ribosome entry site (IRES) and the sequence for enhanced green fluorescent protein (eGFP). The effect of the various 5'UTRs on the translation of sequences downstream will be assessed via retroviral infection of bone marrow (BM) cells (see Figure 3.12 for details of the constructs to be used). Retroviruses encoding the various transgenes will be produced via transient transfection of Bosc23 packaging cells. The virus containing supernatants will be recovered, titred and infection of 5-fluoruracil (FU)-treated BM will be carried out. These BM cells will then be injected into sublethally irradiated syngeneic recipient mice. Bone marrow, thymocytes, splenocytes and LN cells can then be harvested from these mice and analysed by Flow cytometry. In addition, the various 5'UTR- $p56^{Lck}$ cDNA-GFP sequences will be cloned into the $p56^{Lck}$ proximal promoter and transgenic mice will be generated and analysed. These two approaches will enable the effect of the various deletions of the 5'UTRs to be assessed. The efficiency of translation form these
Figure 3.13: Schematic representations of the 5′UTR-p56Lck retroviral constructs

A: Wild-type 5′UTR

\[
\text{LTR} \quad 5′\text{UTR} \quad \text{p56}^{Lck} \quad \text{IRES} \quad \text{GFP} \quad \text{LTR}
\]

B: Deleted 5′UTR

\[
\text{LTR} \quad \text{p56}^{Lck} \quad \text{IRES} \quad \text{GFP} \quad \text{LTR}
\]

C: Mutated 5′UTR

\[
\text{LTR} \quad 5′\text{UTR} \quad \text{p56}^{Lck} \quad \text{IRES} \quad \text{GFP} \quad \text{LTR}
\]

D: pLck 5′UTR

\[
\text{LTR} \quad 5′\text{UTR} \quad \text{p56}^{Lck} \quad \text{IRES} \quad \text{GFP} \quad \text{LTR}
\]

The above diagram shows the structures of the retroviral vectors to be used to transduce murine bone marrow. The LTRs and vector backbone for all of the constructs was MSCV2.2, from the MSCV MIGR1 construct (Pear et al., 1998). The four different 5′UTRs to be investigated are depicted. The stars denote the location of AUG codons, with red stars representing those found in good ribosomal sequence context. Of these, the bona fide initiation codon is indicated with AUG. Green circles indicate AUGs that have been mutated to CUGs. Construct D includes the 5′UTR sequence as present in the p56Lck proximal promoter expression cassette used to generate transgenic mice (see chapters 4 and 5 and refer to Figure 4.1 for sequence details).

constructs can be assessed by analysis of GFP expression. If alterations in the 5′UTR result in altered expression of GFP, and also p56Lck, the effect of this on thymocyte development can be assessed. If the upstream AUGs are important for the control of p56Lck expression then their removal may allow the effect of inappropriate expression of p56Lck within DN1 and DN2 to be assessed.

3.5.2. Alternative mechanisms of translational control

Translation initiation is a frequently targeted step for translational control. One important factor in deciding the rate of translation initiation is the access of mRNA to ribosomes. For example, cell lines derived from patients with multiple melanoma (MM) express ten to twenty-five times more c-myc than
control cell lines (Paulin et al., 1996). This overexpression resulted from aberrant translational control of the \textit{c-myc} gene. This translational control normally functions at the level of mRNA access to ribosomes, since MM cell lines contained over three times as much mRNA in association with polysomes than control cells. It is possible that the \textit{p56}\textsubscript{Lck} transcripts present in DN1 and DN2 are devoid of ribosomes and so are translationally repressed. To this end polysome profiling will be carried out (for discussion and technical details see Garcia-Sanz et al., 1998). This technique allows mRNAs to be fractionated into the soluble, ribonuclear protein fraction (mRNP) and polysome bound fraction. Cells from the four DN subpopulations will be sorted and the total cytoplasmic RNA fractionated in sucrose gradients, Poly(A)+ RNA will then be prepared from the mRNP and ribosome-bound fractions. The RNA samples will then be analysed by northern blotting to allow the proportions of \textit{p56}\textsubscript{Lck} transcripts in the different fractions to be assessed. The low numbers of DN cells obtained by cell sorting has hampered preliminary attempts at polysome profiling. These experiments will be repeated but the extracts from each DN subset obtained from individual sorts will be pooled prior to fractionation in sucrose gradients.

With respect to the potential mechanisms of translational control it is important to mention that although Northern blot analysis has demonstrated the presence of similar levels of \textit{p56}\textsubscript{Lck} transcripts in all four DN subsets, this analysis does not distinguish between different isoforms of mRNA. The \textit{p56}\textsubscript{Lck} transcripts present in DN1 and DN2 may be qualitatively different from those in DN3 and DN4 cells. It is possible that the \textit{p56}\textsubscript{Lck} transcripts present in the later DN subsets lack the out of frame AUGs previously described and so are not subjected to translational masking, thus allowing for efficient translation of \textit{p56}\textsubscript{Lck} within these cells. A similar mechanism of translational control of expression of interleukin 12 (IL-12) has been described (Babik et al., 1999). In non-stimulated cells the predominant form of transcript for the p35 subunit of IL-12 was the poorly translated form which contained an additional upstream ATG. Upon stimulation with LPS transcription initiated from altered upstream positions resulting in transcripts not containing this upstream ATG which showed
enhanced translation. It is possible that signals received by thymocytes later in
development also result in transcription of p56Lck being initiated from altered
positions, thus resulting in different transcripts.

Northern blotting allows for the distinction of transcripts of different
sizes, but in my hands the resolution is limited to approximately 100 base pairs.
In this context, previous work has demonstrated the presence of three different
p56Lck transcripts originating from the proximal promoter within peripheral
blood lymphocytes (PBLs) from normal patients and leukaemic patients (Rouer
et al., 1994). These transcripts were found to represent differently spliced
transcripts, differing only in the sequence of the 5'UTR, which should encode
for the same protein. The Type 1A transcripts included the full-length 5'UTR,
within which two upstream AUGs were identified. The Type 1B and Type 1C
transcripts are 77 base pairs and 98 base pairs shorter than the Type 1A
transcript respectively, and include only one of the upstream AUGs.
Interestingly, the Type 1B transcript is always more abundant than the Type 1A
transcripts in T cell lines and mature T cells from PBL. The level of Type1B
transcripts was significantly increased in PBLs from leukaemic patients in
comparison to normal patients. A similar mechanism of alternate splicing
between cryptic and acceptor donor sites within the 5'UTR of p56Lck transcripts
may occur within the DN thymocytes, resulting in differential expression of
mRNA in DN1 and DN2 cells in comparison to DN3 and DN4 thymocytes. The
heterogeneity of 5'UTRs of the p56Lck transcripts will be analysed. Rapid
amplification of cDNA ends (5'-RACE) will be performed using the SMART-
RACE cDNA Amplification Kit (Clontech). The 5' ends of all p56Lck transcripts
can be sub-cloned and sequenced from each DN subset. RACE (using the
"SMART" protocol) utilises the fact that when certain MMLV-RT enzymes reach
the end of an RNA template they exhibit a terminal transferase activity that
adds 3-5 dC residues to the 3' end of the cDNA. The SMART oligonucleotide
(with a terminal stretch of dG residues) can anneal to the dC rich tail and serve
as an extended template for RT, resulting in production of a full cDNA copy of
the original cDNA with the additional SMART sequence at the end. Further
PCR with the SMART oligonucleotide and an antisense primer specific for
sequence within the first 100 base pairs of p56<sup>Lck</sup> coding sequence will then allow for the generation of 5′-RACE products that can be cloned and sequenced. In this way the presence of qualitatively distinct p56<sup>Lck</sup> transcripts at different developmental stages can be analysed.

Depending on the results obtained this work could be extended. In addition to the presence or absence of upstream AUGs, other features of the 5′UTR of transcripts can effect the efficiency of translation. The length of the 5′UTR and formation of secondary structures within this region can effect the rate of translation initiation rates and so this will also be investigated (Kozak 1989; Kozak 1991; Sagliocco et al., 1993). In addition to regulation by secondary structure, ribosome binding can also be affected by the binding of proteins to the 5′UTR (for review see Sonenberg 1994). For example, the translation of ferritin mRNA is controlled in this way. Ferritin functions in cells to sequester iron from the cytoplasm and its expression is controlled by iron levels. Ferritin mRNA has an iron response element (IRE) within the 5′UTR which folds into a stem-loop structure and to which an IRE binding protein (IRE-BP) binds resulting in repression of translation (Klausner et al., 1993). The affinity of the IRE-BP for the IRE is regulated by iron. When iron is abundant the regulatory protein has low affinity for the IRE and translation occurs normally. However, when iron is limiting the increased affinity of this regulatory protein represses ferritin translation. The translation of other transcripts, including TGF-β mRNA (Romeo et al., 1993), is also inhibited by binding of cytosolic proteins to regions of secondary structure within the 5′UTRs. Further work investigating the differential expression of genes in the DN1 and DN2 cells in comparison to the DN3 and DN4 thymocytes may allow putative translation-inhibiting proteins to be identified.
Chapter 4

Generation of pLck-GFP transgenic mice

4.1. Introduction

Analysis of the expression of p56Lck protein and transcripts during thymocyte development revealed a significant up-regulation of protein level as cells progress into the CD44'CD25+(DN3) thymocyte subset, but a more uniform pattern of mRNA expression. This pattern of regulation is mediated, at least in part, by sequences within the proximal promoter of the p56Lck loci (Garvin et al., 1988; Allen et al., 1992). The p56Lck proximal promoter is commonly used to express transgenes at a high level specifically in thymocytes. As well as directing the expression of the p56Lck gene in a tissue specific manner, these proximal promoter sequences can also drive the expression of heterologous genes (Chaffin et al., 1990; Garvin et al., 1990; Wayne et al., 1994a; Wayne et al., 1994b; Galandrini et al., 1997). To investigate how closely this promoter recapitulates the endogenous regulation of p56Lck gene expression, several independent founder lines expressing a green fluorescent protein (GFP) reporter under the control of the p56Lck proximal promoter were generated.

4.1.1. Green fluorescent protein

The autofluorescent protein GFP was discovered as a companion protein to aequorin, the original bioluminescent phosphoprotein from the jellyfish Aequorea victoria (Shimomura et al., 1962). The gene for GFP was first cloned in 1992 and, following the demonstration of its expression in non-jellyfish systems, has become a versatile tool for the study of gene expression and protein localisation, through protein tagging experiments (Prasher et al., 1992; Chalfie et al., 1994; Misteli and Spector 1997). Since GFP does not require unique jellyfish factors to fluoresce it is useful in many cell types, from numerous different organisms including bacteria, nematodes, fish, and mice (Chalfie et al., 1994; Amsterdam et al., 1995; Ikawa et al., 1995). GFP is a stable protein, its spectral properties are unaffected by many detergents, proteases and ranges of
temperature and pH (for review see Tsien 1998). The crystal structure of GFP has recently been solved helping to explain its stability. The protein consists of eleven β-sheets surrounding a central α-helix, which contains a chromophore (Ormo et al., 1996). In the jellyfish, GFP is activated in a calcium-dependent manner, when calcium binds to another bioluminescent protein, aequorin, which transfers energy indirectly to GFP and triggers the release of green light. This energy transfer can be mimicked experimentally by simple exposure of GFP to blue excitation light (wavelength of 488nm), resulting in emission of green light. Thus, GFP expressing cells can be easily identified using a fluorescent microscope or a Flow cytometer.

Random and site directed mutagenesis has produced potentially useful mutants of wild-type GFP, with single and multiple amino acid substitutions that exhibit different excitation and emission spectra (for a review of GFP variants see Tsien 1998). The GFP cDNA used here (kind gift from Dr Jonathon Pines) incorporates a number of mutations that improve its spectral properties and enhance its use as a marker in murine cells (Zernicka-Goetz et al., 1996; Zernicka-Goetz et al., 1997). One distinct disadvantage of wild-type GFP is that although it folds efficiently when expressed at or below room temperature, its folding efficiency decreases significantly at higher temperatures. The GFP cDNA used here includes three mutations that improve the folding of the protein, enhancing its solubility at 37°C (Cormack et al., 1996). These changes increase the stability of the protein and the brightness of its fluorescence. Wild-type Aequorea victoria GFP has a complex spectra of activities. It has a major excitation peak at 395nm and a minor peak at 475nm. An additional mutation (Ser65Thr) in the GFP to be used here, enhances the peak of excitation at the longer wavelength, which is of more use experimentally since this matches with standard FITC filter sets (Heim et al., 1995). Previous work has demonstrated that this GFP is easily visible and non toxic to mouse cells and does not perturb embryogenesis (Zernicka-Goetz et al., 1996; Zernicka-Goetz et al., 1997). This chapter presents an analysis of transgenic mice in which GFP cDNA is expressed under the control of the p56Lck proximal promoter, to investigate the expression pattern of this promoter when driving a heterologous gene.
4.2. Specific Methods

4.2.1. pLckGFP Transgenic construct

The 700 base pair GFP cDNA, which includes a translational start site and a stop codon, was PCR amplified from pCMX-F64GFP (Zernicka-Goetz et al., 1996) with primers GFP1 and GFP2 (see below). The PCR product was then digested with Kpn1 and BamH1 and blunt cloned into the BamH1 cloning site of the \( p56^{Lck} \) proximal promoter expression cassette (see Figure 4.1, page 112). This cassette contains the \( p56^{Lck} \) proximal promoter and introns and exons of the human growth hormone (hGH) gene to enhance the expression of the transgene (Chaffin et al., 1990). The presence of these introns is thought to improve the efficiency of expression of the transgene, possibly by enhancing the processing and transport of the transgenic mRNA to the cytoplasm (Brinster et al., 1988; Palmiter et al., 1991). As shown in Figure 4.1, ninety-seven amino acids from the 5' untranslated region (5'UTR) of endogenous \( p56^{Lck} \) transcripts are maintained in the \( p56^{Lck} \) proximal promoter expression cassette used here. The transgene was excised with Not1 prior to injection. Transgenic mice were identified by hybridisation of EcoR1 digested genomic Southern blots with a transgene specific hGH probe.

GFP1: 5'- cgcggatccagggagaacaaagctgggtac-3'
GFP2: 5'- ctaggtgatcaagatctcgccggc-3'

4.2.2. Typing of pLck-GFP transgenic mice via PCR

Once founder lines had been established transgenic mice were typed using PCR. The primers shown below (GFP3 and GFP4) were used. GFP3 and GFP4 are specific for sequences in the 5' and 3' end of the GFP cDNA respectively. These primers generate a 540 base pair product. The sequence between these two primers was also used as a GFP-specific probe for the detection of GFP mRNA on Northern blots.

GFP3: 5'-tggagagggtgaaggtgatgc-3'
GFP4: 5'-tgtgtggacaggtaatggttg-3'

PCR conditions were as described in section 2.7.
Cycles were as follows:

First cycle: denaturation at 94°C for 4 minutes
annealing at 54°C for 40 sec
extension at 72°C for 40 sec

Subsequent 39 cycles:
denaturation at 94°C for 30 sec
annealing at 54°C for 40 sec
extension at 72°C for 40 sec

Final extension period: 72°C for 5 minutes

4.2.3. Immunofluorescence analysis by flow cytometry and cell sorting

Single cell suspensions of thymocytes, splenocytes and LN cells were prepared as previously described. Cell suspensions were filtered and stained for the detection of surface antigens and then analysed by flow cytometry. Due to the presence of GFP, which emits at approximately the same frequency as FITC-conjugated antibodies, these conjugates could not be used. Therefore, when required, four colour flow cytometric analysis and cell sorting were performed on the FACSCalibur and FACS Vantage respectively (Becton Dickinson). Monoclonal antibodies conjugated with phycoerythrin (PE), biotin (followed by second layer of streptavidin-Tricolour) and allophycocyanin (APC) were used. PE and TC fluorochromes are excited by the argon-ion laser (emits light at 488nm) and APC fluorochromes are excited by the helium-neon laser (emits light at 630).

4.2.4. Fluorescence microscopy analysis of unstained GFP thymocytes

Thymocytes were centrifuged at 1000 rpm onto polylysine-coated coverslips (100μg/ml polylysine in water, Sigma). The coverslips were then mounted onto microscope slides with 5μl polyvinyl alcohol (PVA) based Mowiol 4-88 (Hoechst, Frankfurt) mounting medium (prepared as previously described Osborn and Weber 1982). Cells were analysed on a Zeiss Axiovert Fluorescence microscope under oil immersion, with a 40x objective lens and a numerical aperture of 1.3. Images were captured using IP Lab Spectrum software (Sigma Analytics Corporation, Vienna).
4.3. Results

4.3.1. Generation of transgenic mice expressing Green Fluorescent Protein (GFP) under the control of the p56Lck proximal promoter

The pLck-GFP construct was generated as described in section 4.2.1. (Figure 4.1). The transgene was excised with NotI prior to pronuclear injection into fertilised (CBA/Ca x C57BL/6-J) F1 oocytes. Transgenic founders were identified by Southern blot hybridisation of genomic DNA extracted from tail snips and digested with EcoRI. EcoRI cuts at the 5' and 3' end of the cloned transgene removing a 6.0kb fragment that can be detected by hybridisation with the human growth hormone.

In total, six founders were identified out of one hundred and forty mice screened. Figure 4.2A shows a southern blot identifying 4 of these founders. The relative copy number of each of the lines was estimated by stripping the blots and re-hybridising with a probe specific for CBFα1, an endogenous gene present at a single locus. Using a phosphorimager for quantitation, it was estimated that founder LG1-1 had a relative copy number of 1 and founders LG-2, LG-3 and LG-4 had relative copy numbers of 4, 20 and 2 respectively. These founders were bred with C57BL/6-J mice, generating lines LG-1, LG-2, LG-3 and LG-4. The transgenic lines were stable and capable of germline transmission of the transgene. Figure 4.2B shows a representative litter of six from founder line LG-1. Following initial identification of founders and establishment of founder lines, transgenic mice were identified using PCR (Figure 4.3 and see section 4.2.2 for details of the primers used).

4.3.2. Analysis of GFP expression in pLck-GFP transgenic thymocytes

In order check for the expression of the pLck-GFP transgene within thymocytes fluorescence microscopy was initially used. Thymocytes were isolated from transgenic and non-transgenic littermates from line LG-1 and were analysed on a fluorescence microscope for the expression of GFP. As shown in Figure 4.4, GFP fluorescence is detected within unstained pLck-GFP thymocytes, demonstrating expression of the pLck-GFP transgene.
Figure 4.1: Generation of pLck-GFP transgenic mice

The pLck-GFP transgenic construct comprises the \( p_{56^{lck}} \) proximal promoter (-3200 to +97 with respect to the transcription start site) fused to the GFP cDNA (Zernicka-Goetz et al., 1996). The sequence of the full 5' untranslated region (5'UTR) of \( p_{56^{lck}} \) transcripts derived from the murine proximal promoter is provided below. The transcription start site (designated +1) and the \textit{bona fide} initiation codon (boxed) are shown. Upstream AUG codons present in the 5'UTR are indicated in bold and with the one described as being in good Kozak coding sequence is underlined (Marth et al., 1988; Perlmutter, 1990). The 5'UTR sequence present in the pLck-GFP cassette is presented inside the blue-hashed boxes. A polyadenylation signal (pA+) and sequences for the human growth hormone (hGH) gene were added to the 3'end to enhance splicing and expression of the transgene (Chaffin et al., 1990). The transgene was excised with NotI prior to injection. Transgenic mice were identified by hybridisation of EcoR1 digested genomic Southern blots with a transgene-specific, previously characterised hGH probe.

```
AUG TAA
0.7Kb
TAA
NotI
EcoR1
pLck proximal promoter
3.2Kb

AUG GAGGGAACCCAGTCAGGAGCTTGAATCCCACGATTCAGCGCTTCT
GTCTGGGCGCAATGGGGGCCTCTGAGCrGACG-ATCTCGGGTACTT
TTTGTAA pxcCAGA ACAGGGCTCTAGGA T G TCTG A TG TTG G G G CG
AGTGGCTTAGGGCCAGCTCCTTCAGGCCTCTCTA-CATTCCTTCAGG

GATC\textbf{ATG}

```
Figure 4.2: Southern blot analysis of pLck-GFP transgenic mice

A: Transgenic pLck-GFP founders were detected by hybridisation of EcoR1 digested genomic Southern blots with the transgene-specific human growth hormone probe. Four pLck-GFP founders were detected, each with transgene bands at 6Kb. The blots were then stripped and re-hybridised with a probe specific for CBFα1, an endogenous gene present at a single locus, to allow relative copy number (RCN) to be estimated. These founders were backcrossed with C57Bl/6 mice to establish transgenic lines LG-1, LG-2, LG-3 and LG-4.

B: Germline transmission was confirmed for all four lines. The Southern blot below shows a representative litter of six from line LG-1 with four transgenic and two non-transgenic offspring.
Figure 4.3: PCR genotyping of pLck-GFP transgenic mice

Following initial identification of founders and establishment of founder lines, transgenic mice were identified using PCR. The primers and conditions used are described in section 4.2.2. A “+” indicates a transgenic and a “-” indicates a non-transgenic littermate. A control reaction with distilled water (DW) instead of DNA was also performed.
Figure 4.4: Thymocytes from pLck-GFP transgenic mouse line LG-1 demonstrating GFP expression
No fluorescence was detected in unstained thymocytes from non-transgenic littermate control mice.

To further investigate the expression of the pLck-GFP transgene, thymocytes, LN cells and splenocytes were isolated from mice from each founder line, stained with monoclonal antibodies and analysed by flow cytometry. In founder line LG-1, GFP fluorescence was detected in over 90% of thymocytes, 75% of peripheral lymph node cells and 30% of splenocytes (Figure 4.5A). The cells of the lymph node and spleen that expressed GFP were shown to be T cells by staining with anti-Thy-1 (Figure 4.5B). No B cells or macrophages expressed GFP. Approximately 75% of γδ T cells and 50% of NK (natural killer) T cells (positive for the cell surface marker NK1.1 and CD3) contained GFP.

To assess the expression of GFP throughout T cell development thymocytes were stained with immunofluorescent antibodies against cell surface maturation markers CD4, CD8, CD25 and CD44. In founder line LG-1 the early double negative (DN) compartments, CD44⁺CD25⁻ (DN1) and CD44⁺CD25⁺ (DN2) cells, lacked GFP fluorescence (Figure 4.6). The later DN subsets expressed GFP, with 46% of CD44⁺CD25⁺ (DN3) cells and 64% of CD44⁺CD25⁻ (DN4) cells being positive for GFP. Over 90% of CD4⁺CD8⁺ DP, CD4⁺CD8⁻ SP and CD4⁺CD8⁺ SP cells expressed GFP. Thus, the expression pattern of GFP in this founder line is comparable to the pattern of p56^{Lck} protein expression in non-transgenic mice.

The pattern of GFP expression in the other founder lines was also analysed as described above. Thymocytes, peripheral lymph node cells and splenocytes from each of the four independent lines expressed GFP (Figure 4.7). These GFP positive cells were T cells, as demonstrated by staining with anti-Thy1 (data not shown). GFP was expressed in γδ T cells and NK T cells. Few B cells or macrophages expressed GFP. Although the profile of GFP expressing cells was similar in each founder line analysed, the percentage of expressing cells varied in each line. Thus, GFP expression was confined to the T cell lineage, in all four independent lines, confirming the T cell specificity of the p56^{Lck} proximal promoter.
Figure 4.5A: Thymocytes, lymph node cells and splenocytes from pLck-GFP founder line LG-1 were isolated and stained with monoclonal antibodies prior to flow cytometric analysis. The percentage of cells expressing GFP, and falling within the positive marker is given. This marker was set to exclude at least 99% of GFP negative thymocytes from a non-transgenic control mouse. The results are representative of five different mice.

![Flow cytometry histograms showing GFP expression in thymocytes, lymph node (LN) cells, splenocytes, B cells, NK T cells, γδ cells, and macrophages.](image)

Figure 4.5B: Lymph node cells and splenocytes from the above experiment were stained with an anti-Thy1.1 antibody

![Flow cytometry plot showing Thy1.2 expression in GFP-negative and GFP-positive cells in LN cells and splenocytes.](image)
Figure 4.6: GFP expression in pLck-GFP line LG-1 during thymocyte development

Thymocytes from pLck-GFP founder line LG-1 were isolated and stained with monoclonal antibodies prior to flow cytometric analysis. The percentage of cells expressing GFP, and falling within the positive marker is given. This marker was set to exclude at least 99% of GFP negative thymocytes from a non-transgenic control mouse. The results are representative of five different mice.
Figure 4.7: Comparison of pLck-GFP protein expression in four independent founder lines

The expression of the pLck-GFP transgenic protein in four independent founder lines was analysed by flow cytometry. The percentage of cells expressing GFP in each cell type is shown. Data presented is an average taken from four sets of experiments for each founder line. The relative copy number (RCN) of each founder line is shown. Abbreviations used: T, thymocytes; LN, lymph node cells; S, splenocytes; B, B cells; G, γδ T cells; N, NK T cells; M, macrophages.
In contrast to the broad similarity of GFP expression in T cells, B cells and macrophages, there was a significant degree of variation in GFP expression in the early DN thymocyte compartment between the four transgenic lines (Figure 4.8). In founder line LG-1, the DN1 and DN2 subsets lacked detectable GFP fluorescence whereas over 50% of cells of the more mature DN subsets expressed GFP. In founder line LG-3, the DN1 subset lacked GFP fluorescence, but nearly 20% of DN2 thymocytes and over 40% of cells from the DN3 subset onwards expressed GFP. The expression pattern seen in founder line LG-4 was similar to that seen in founder line LG-3, except that about 5% of both DN1 and DN2 thymocytes showed GFP fluorescence and other subsets expressed GFP in no more than 50% of cells. In founder line LG-2 over 80% of all DN, DP and SP thymocytes expressed GFP (note that this line does not have the highest relative transgene copy number). There is significant variation in the proportion of cells expressing GFP within different thymocyte subsets in independent founder lines.

4.3.3. Northern blot analysis of the expression of the pLckGFP transgene

In addition to the above analysis of the expression of the GFP transgene at the protein level, the expression of this transgene was also investigated at the RNA level. Cells from the four DN thymocyte subsets were isolated and total RNA was extracted, as previously described. To obtain $2 \times 10^5$ cells for each DN thymocyte subset up to twenty-five mice are needed. For this reason these Northern blotting experiments were carried out with thymocytes from just two of the lines (LG-1 and LG-2). RNA was also extracted from GFP positive cells (i.e. those that express GFP as a protein) and GFP negative cells (i.e. those that do not express GFP as a protein) from transgenic and non-transgenic mice respectively, to verify the specificity of the probe. GFP mRNA was expressed in all four DN populations at a relatively constant level in both lines (Figure 4.9). GFP mRNA was present in the GFP positive cells and absent from the GFP negative cells. Thymocytes from the two independent founder lines analysed express a similar level of GFP mRNA. This is surprising considering the different relative copy numbers (RCN) of these two lines (line LG-1, RCN of 1, line LG-2, RCN of 4) and the varying percentage of cells expressing GFP protein in these two lines.
4.8: Comparison of GFP protein expression during thymocyte development in four independent founder lines

The expression of the pLck-GFP transgenic protein during thymocyte development in four independent founder lines was analysed by flow cytometry. The percentage of cells expressing GFP in each cell type is shown. Data presented is an average taken from four sets of experiments for each founder line. The relative copy number (RCN) of each founder line is shown. Abbreviations used: 1, Double negative (DN) 1 cells; 2, DN2 cells; 3, DN3 cells; 4, DN4 cells; DP, double positive thymocytes; SP, single positive thymocytes.
Figure 4.9: Northern blot analysis comparing the expression of GFP mRNA in thymocytes from line LG-1 and LG-2

Total RNA was prepared from $2 \times 10^5$ cells from the DN thymocyte subsets from pLck-GFP founder lines LG-1 and LG-2. The relative copy number (RCN) of each founder line is shown. The RNA samples were electrophoresed on formaldehyde-agarose gels and the blot was hybridised with the indicated probes. The intensity of the GFP band was quantitated using a phosphorimager and the values were normalised to $\beta$-actin rather than GAPDH because of the similarity in size of GFP and GAPDH transcripts. $10^6$ GFP positive cells (GFP+) and $10^6$ GFP negative (GFP-) cells were used as controls. Abbreviations used: 1, double negative (DN)1 cells; 2, DN2 cells; 3, DN3 cells; 4, DN4 cells.

<table>
<thead>
<tr>
<th>Line LG-2 (RCN 4)</th>
<th>Line LG-1 (RCN 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4</td>
<td>1 2 3 4 GFP+ GFP-</td>
</tr>
</tbody>
</table>

**GFP** (1.6Kb)

**$\beta$-Actin** (2.2Kb)

Relative quantitation for GFP

| 1.0 | 1.4 | 1.1 | 1.5 | 0.7 | 0.9 | 0.9 | 1.1 |
GFP mRNA is therefore expressed throughout thymocyte development at a relatively constant level, even in thymocytes from line LG-1 which do not express GFP as a protein until the DN3 subset. This mirrors the result seen for the expression of endogenous p56Lck mRNA previously discussed (Figures 3.6 and 3.9).

Figure 4.9 indicated that the relative copy number of the transgene might not affect the level of expression of GFP transcripts, since lines LG-1 and LG-2 expressed similar levels of GFP mRNA, despite having transgene RCNs of 1 and 4 respectively. This result suggests that the p56Lck proximal promoter may not exhibit locus control region (LCR) activity. LCRs are defined as gene regulatory sequences that confer copy number-dependent expression of a transgene, independent of its chromosomal position of integration (Grosveld et al., 1987). LCRs may act by establishing, and/or maintaining, a stable open chromatin structure of a locus, thus ensuring that regardless of the normal degree of chromatin condensation at the site of integration, transcription factors can access the necessary regulatory elements and direct transgene expression. LCRs are found in close proximity to, and as far as 100Kb from, transcribed sequences. The LCR of the human β-globin locus was the first to be identified, through studies with transgenic mice expressing only the human β-globin gene without upstream sequences and investigations into human thalassemias where the β-globin genes are intact but the region upstream of the locus is deleted (Kioussis et al., 1983; Magram et al., 1985). Additional work has further characterised the LCR of the human β-globin gene and of other genes, including the human CD2 gene and the murine CD4 gene (Grosveld et al., 1987; Greaves et al., 1989; Boyer et al., 1997). To determine whether the p56Lck proximal promoter exhibited LCR activity, GFP steady state RNA expression was determined in each of the founder lines. As shown in Figure 4.10, the levels of GFP transcripts, as detected by Northern blotting analysis, were not predicted by the copy number of the transgene. For example, Founder line LG-3 has a RCN of 20 and yet thymocytes from these mice express a similar level of GFP transcripts as cells from founder line LG-1 that has a RCN of 1. These data provide no evidence for LCR activity within the p56Lck proximal promoter.
Figure 4.10: Northern blot analysis of the comparative level of *GFP* transcripts in cells from different pLck-GFP founder lines

Total RNA was prepared from $5 \times 10^5$ total thymocytes from each of the four founder lines. The RNA samples were electrophoresed on formaldehyde-agarose gels and the blot was hybridised with the indicated probes. The intensity of the *GFP* band was quantitated using a phosphorimager and the values were normalised to *β-Actin*. The relative copy number (RCN) for each founder line is indicated.
Previous studies have concluded that \( p56^{Lck} \) gene expression is developmentally regulated by two independent promoters. The distal promoter has been described as predominating in mature single positive (SP) T cells, while the proximal promoter as mainly active in developing thymocytes (Garvin et al., 1988; Reynolds et al., 1990; Wildin et al., 1991; Allen et al., 1992). To further investigate the activity of the proximal promoter, the expression of \( GFP \) mRNA within thymic and peripheral CD4 and CD8 SP cells was analysed. \( GFP \) mRNA was present within CD4 SP and CD8 SP cells from the thymus and from the periphery (Figure 4.11). When normalised to the level of \( \beta\text{-}Actin \) mRNA, \( GFP \) was expressed at the same level in CD4 SP thymocytes as in CD8 SP thymocytes. The level of \( GFP \) expression in peripheral T cells was also the same in CD4 SP cells as CD8 SP cells. \( GFP \) was expressed at a marginally lower level within peripheral cells in comparison to thymocytes. The presence of \( GFP \) mRNA within CD4 and CD8 SP cells from the periphery suggests that the \( p56^{Lck} \) proximal promoter is active in these T cells. Thus, the protein previously detected in peripheral T cells from the spleen and lymph nodes of transgenic thymocytes may represent \textit{de novo} protein synthesis, not simply stable protein made earlier in the development of the cell.

4.3.4. \textit{Fluorescence in situ} hybridisation analysis of the Lck-GFP transgenic lines

As previously discussed, there is no evidence for LCR activity in the \( p56^{Lck} \) proximal promoter. Studies have demonstrated that expression of transgenes downstream of promoters with incomplete or absent LCR activity can be subject to chromosomal position effects due to the random site of integration (Festenstein et al., 1996; Milot et al., 1996; Boyer et al., 1997). Thus, other factors relating to the integration site of the transgene may have important affects on the expression of transgenes downstream of this promoter. The location of the pLck-GFP transgene in each founder line was investigated by \textit{fluorescence in situ} hybridisation (FISH) using a probe specific for \textit{human growth hormone} sequences within the transgene. The chromosomes were counter-stained with 4',6-diamidino-2-phenyindole (DAPI) and chromosomal paints to enable the
Figure 4.11: Northern blot analysis of the expression of GFP in within single positive cells from the thymus and the spleen from Founder line LG-1

Total RNA was prepared from 2 x 10^5 cells from CD4 and CD8 single positive cells from the thymus and the spleen. The RNA samples were electrophoresed on formaldehyde-agarose gels and the blot was hybridised with the indicated probes. The intensity of the GFP band was quantitated using a phosphorimager and the values were normalised to β-Actin. 10^6 GFP positive and 10^6 GFP negative cells were used to test the specificity of the GFP probe. Abbreviations used: T, thymus; S, spleen.

<table>
<thead>
<tr>
<th></th>
<th>CD4 (T)</th>
<th>CD4 (S)</th>
<th>CD8 (T)</th>
<th>CD8 (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GFP</strong> (1.6Kb)</td>
<td><img src="image" alt="GFP Band" /></td>
<td><img src="image" alt="GFP Band" /></td>
<td><img src="image" alt="GFP Band" /></td>
<td><img src="image" alt="GFP Band" /></td>
</tr>
<tr>
<td><strong>β-Actin</strong> (2.2Kb)</td>
<td><img src="image" alt="β-Actin Band" /></td>
<td><img src="image" alt="β-Actin Band" /></td>
<td><img src="image" alt="β-Actin Band" /></td>
<td><img src="image" alt="β-Actin Band" /></td>
</tr>
</tbody>
</table>

Relative quantitation for GFP:

- CD4 (T): 1.2
- CD4 (S): 1.0
- CD8 (T): 1.5
- CD8 (S): 1.4
chromosome to be identified. Various staining methods, such as Giemsa or DAPI staining, allow mouse chromosomes to be distinguished due to the reproducible banding patterns that are accentuated by these dyes. Dark Giemsa-stained bands, called G bands, alternate with Giemsa-negative bands, called R bands or reverse bands. G bands represent condensed regions of heterochromatin and R bands represent regions of euchromatin that appear to be decondensed during interphase (see section 4.4.2 for details).

FISH analysis revealed a single integration site for each transgenic line (Figure 4.12A and B). The insertion site of the transgene was located to chromosome 6, region A2-3 in founder line LG-1; Chromosome 1, region H2-3 in founder line LG-2; Chromosome 2, region H3-4 in founder line LG-3 and chromosome 14, region A2-3 in founder line LG-4. In lines LG-1 and LG-4 the transgenes have integrated relatively close to the centromere and in line LG-2 and LG-3 the transgenes have integrated close to the telomere.

4.3.5. Generation of homozygous pLckGFP mice (Lines LG-1 and LG-3)

All of the pLck-GFP data presented thus far has studied the expression of GFP in heterozygous transgenic mice. It is also of interest to analyse the expression of pLck-GFP transgenes in homozygous transgenic mice since these should express a higher level of the transgene. Lines LG-3 and LG-4 show similar transgene expression patterns so only one line was chosen to generate homozygous mice. Heterozygous mice from line LG-2 express GFP in over 80% of all thymocyte subsets, thus any effects of breeding this line to homozygosity would be expected to be minimal. Therefore, an attempt was made to breed founder lines LG-1 and LG-3 to homozygosity. To obtain homozygous mice, heterozygotes were intercrossed. According to Mendelian genetics the F1 (first filial) generation will consist of three different genotypes, 50% will be heterozygous, 25% will be homozygous and 25% will be wild-type. In order to identify the homozygous mice of the F1 generation, mice were backcrossed to C57BL/6 mice, because only homozygous mice give rise to all heterozygous litters in the F2 generation in such a backcross. For each line three intercrosses were set up and all of the F1 offspring were backcrossed and the resultant litters genotyped for transgenic status. Fifteen F1 offspring from line LG-1 were backcrossed but
Figure 4.12: Fluorescence in situ Hybridisation (FISH) analysis of pLck-GFP Transgenic lines

Figure 4.12A: Localisation of Lck-GFP transgenic integration sites by chromosomal FISH to metaphase chromosomes. Transgene insertion was visualised using avidin-Texas Red after hybridisation with a biotinylated probe specific to human growth hormone sequences within the transgene. A diagrammatic representation of the chromosomal location of the transgene (indicated by a green star) on an idealistic mouse chromosome, or idiogram, stained with Giemsa is shown for each transgenic line. The black bands represent dark Giemsa-stained bands (G bands) and white bands represent Giemsa-negative bands (R bands).

Line LG-1

Chromosome 6
region A2-3

Line LG-2

Chromosome 1
region H2-3
Figure 4.12B: Further FISH analysis of pLck-GFP transgenic lines

Localisation of Lck-GFP transgenic integration sites by chromosomal FISH to metaphase chromosomes. A diagrammatic representation of the location of the transgene (indicated by a green star) on an idiogram stained with Giemsa is shown for each transgenic line.
no homozygous mice were detected. The reason for this is unknown but the
generation of homozygous mice can result in deficiency of endogenous genes
present at the locus of integration. This may explain the failure to obtain
homozygotes for this line. Assessment of the transgenic status of sixteen F1 mice
from line LG-3 allowed three homozygotes to be identified. These mice were
intercrossed, to establish a homozygous LG-3 line. The homozygosity of these
mice was confirmed by FISH analysis (Figure 4.13A). The detection of a
transgene signal on two chromosomes, both at region H3-4 on each
chromosome 2, indicated that the mice were homozygous for the pLck-GFP
transgene at this locus.

Expression of the pLck-GFP transgene in thymocytes, lymph node cells
and splenocytes from the LG-3 homozygous mice was analysed by flow
cytometry. As shown in Figure 4.13B, the percentage of GFP expressing cells in
the homozygous mice had increased significantly in comparison to the
heterozygous mice. The percentage of total thymocytes expressing GFP had
increased from 52% to 83% in the homozygous mice. In all subpopulations
investigated the percentage of transgene positive cells had increased. Thus, the
presence of the pLck-GFP transgene on both chromosomes, at a single loci,
rather than just on one chromosome increases the percentage of transgene
expressing GFP.

4.3.6. Summary

To investigate the expression pattern of the p56Lck proximal promoter
founder lines expressing a GFP reporter under the control of this T cell specific
promoter were generated. Transgene expression in four independent pLck-GFP
founder lines, one of which was bred to homozygosity, has been described.
These analyses included investigations into the relative copy number (RCN) of
each line, the chromosomal site of integration of the transgene, the percentage of
cells expressing the transgene and the relative level of expression of the
transgene. Table 4.1 summarises the relationship between these factors. As
discussed, there is no evidence for LCR activity in the p56Lck proximal promoter,
since the steady state levels of GFP transcripts, as detected by Northern blotting
analysis, were not predicted by the copy number of the transgene.
Figure 4.13: Analysis of homozygous pLck-GFP transgenic mice from Founder line LG-3

4.13A: FISH analysis: Localisation of Lck-GFP transgenic integration sites by by chromosomal FISH to metaphase chromosomes. The detection of a signal on two chromosomes indicates the mouse is homozygous for the transgene.
4.13B: Flow cytometric analysis of the expression of the Lck-GFP transgene in homozygous mice from line LG-3

The percentage of cells expressing GFP in each cell type is shown. Data is representative of three sets of experiments. Abbreviations used: T, thymocytes; LN, lymph node cells; S, splenocytes; B, B cells; G, γδ T cells; N, NK1.1 positive cells; M, macrophages; 1, Double negative (DN) 1 cells; 2, DN2 cells; 3, DN3 cells; 4, DN4 cells; DP, double positive thymocytes; SP single positive thymocytes.
There is also no obvious link between RCN of the transgene and the percentage of GFP expressing cells or the level of GFP fluorescence shown by these cells. This data suggests that factors other than the RCN of a transgenic line, relating to the integration site of the transgene, may have important affects on the expression of transgenes downstream of \( \text{p56}^{\text{Lck}} \) proximal promoter.

**Table 4.1: Comparison of pLck-GFP founder lines**

The four pLck-GFP founder lines were compared with respect to the relative copy number (RCN), the percentage of total thymocytes expressing GFP protein, the relative steady state level of GFP mRNA, the mean GFP fluorescence of GFP expressing thymocytes and the integration site of the transgene.

<table>
<thead>
<tr>
<th>Line</th>
<th>RCN</th>
<th>Percentage of GFP positive thymocytes</th>
<th>Steady state GFP mRNA level of total thymocytes</th>
<th>Mean GFP fluorescence of GFP expressing thymocytes</th>
<th>Location of Transgene</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG-1</td>
<td>1</td>
<td>93.0</td>
<td>2.2</td>
<td>714</td>
<td>Close to centromere</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome 6, region A2-3</td>
</tr>
<tr>
<td>LG-2</td>
<td>4</td>
<td>96.0</td>
<td>1.1</td>
<td>466</td>
<td>Close to telomere</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome 1, region H2-3</td>
</tr>
<tr>
<td>LG-3</td>
<td>20</td>
<td>50.5</td>
<td>1.9</td>
<td>650</td>
<td>Close to telomere</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome 2, region H3-4</td>
</tr>
<tr>
<td>LG-4</td>
<td>2</td>
<td>39.5</td>
<td>1.0</td>
<td>374</td>
<td>Close to centromere</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Chromosome 14, region C</td>
</tr>
<tr>
<td>LG-3 Homozygous</td>
<td>40</td>
<td>82.0</td>
<td>2.4</td>
<td>813</td>
<td>Both transgenes integrated into region H3-4, close to telomere of chromosome 2</td>
</tr>
</tbody>
</table>

**4.4. Discussion**

**4.4.1. Insights into the use of the p56\(^{Lck}\) proximal promoter**

The proximal promoter of the p56\(^{Lck}\) gene has been extensively used to drive the expression of transgenes in thymocytes (for examples see Chaffin et al., 1990; Garvin et al., 1990; Abraham et al., 1991a; Abraham et al., 1991b; Allen et al., 1992; Wayne et al., 1994a; Wayne et al., 1994b; Galandrini et al., 1997). The interpretation of the phenotype of transgenic mice is critically dependent upon
the developmental timing and level of expression of the transgene product. In order to determine these parameters, transgenic mice in which the p56\textsuperscript{Lck} proximal promoter has been used to drive the expression of GFP have been generated (Figure 4.1). This autofluorescent protein, produced by the jellyfish \textit{Aequorea victoria} has been widely used as a reporter in the determination of gene expression and protein localisation, through protein tagging experiments (Misteli and Spector 1997). Analysis of several independent transgenic mouse lines in this study has revealed a number of insights into the use of the p56\textsuperscript{Lck} proximal promoter.

In four independent lines, expression was confined to the T cell lineage, confirming the T cell specificity of this promoter (Figure 4.7). As previously discussed, expression of the p56\textsuperscript{Lck} gene is developmentally regulated by two independent promoters. Although the proximal promoter has been described as being principally active in the thymus, the level of activity of this promoter in peripheral T cells is unclear. Early investigations revealed a five to nine fold lower level of transcripts from the proximal promoter in comparison to the distal promoter in mature T cells (Reynolds et al., 1990). Results presented here have detected a high level of GFP fluorescence in peripheral T cells from the spleen and lymph nodes and in N.K T cells. As previously discussed, GFP is a very stable protein, thus, the expression of GFP in mature T lineage cells may reflect their developmental history rather than the inappropriate expression of the transgene (Ormo et al., 1996). However, we have detected GFP transcripts in peripheral T cells at a similar level to thymocytes (Figure 4.11). These studies suggest that the p56\textsuperscript{Lck} proximal promoter does possess a reasonable level of activity at later stages of T cell development. The GFP mRNA present in peripheral T cells detected here may result from transcription occurring whilst these cells were still developing the thymus. Future work aims to investigate this further through adaptation of techniques, such as Nuclear “run off” assays that are routinely used to measure the rate of transcription. This technique involves the isolation of nuclei from the cells of interest and followed by their incubation in the presence of four ribonucleotides, one of them radiolabelled. Total nuclear RNA, including the newly synthesised-labelled RNA, is extracted
and hybridised to a denatured, membrane-bound cDNA corresponding to the mRNA being measured. After hybridisation the membrane is treated with RNase A and washed to remove the non-specifically bound RNA. Autoradiography of the membrane enables newly synthesised specific RNA to be quantitated. This methodology could be adapted here to assess the transcription of novel GFP mRNA in peripheral T cells from the pLck-GFP mice.

The principle aim of these studies was to investigate how closely the \( p56^{Lck} \) proximal promoter cassette recapitulates the endogenous regulation of \( p56^{Lck} \) gene expression when driving the expression of a heterologous gene. Analysis of the DN thymocyte compartment revealed a pattern of GFP expression that varied significantly between independent founder lines. In three of the four lines examined, there was a clear but variable upregulation of GFP protein expression between the DN2 population and the DN3 subset, as was observed for endogenous \( p56^{Lck} \) protein. However, in only one line was there a lack of detectable GFP expression in DN1 and DN2 thymocytes. Furthermore, in one transgenic line over 80% of each DN thymocyte subset, as well as DP and SP thymocytes, expressed GFP. In two lines no more than 60% of any thymocyte population contained GFP. Thus, the endogenous pattern of expression, where greater than 90% of DN3 and DN4 cells but less than 2% of DN1 and DN2 thymocytes express \( p56^{Lck} \) protein, was not faithfully reproduced in any of the transgenic founder lines.

One possible explanation for the differences in expression between the GFP transgene and endogenous \( p56^{Lck} \) is that the sequence of the 5' untranslated region (5'UTR) present in the proximal promoter cassette is not complete (see Figure 4.1). As discussed in Chapter 3, the 5' UTR of endogenous \( p56^{Lck} \) transcripts includes a number of upstream AUG triplets. At least one of these codons has been shown to be in good sequence context according to Kozak's criteria. Mutational analysis of the 5'UTR region of \( p56^{Lck} \) transcripts revealed that removal of these AUG triplets resulted in a seven to ten-fold increase in translation of a reporter gene in comparison to the wild-type construct (Marth et
Therefore, p56\textsuperscript{Lck} transcripts normally contain a 5'UTR that reduces the efficiency of translation, possibly due to translational masking. The proximal promoter cassette contains only half of this 5'UTR sequence and importantly does not include the AUG thought to be in good Kozak sequence context. Therefore, the putative translational control mechanism regulating p56\textsuperscript{Lck} expression in T cells may not be operative in the GFP transgenic mice. In founder line LG-1 the expression pattern of GFP is in broad agreement with endogenous expression of p56\textsuperscript{Lck} protein in normal mice. GFP transcripts are present in all four DN thymocyte subsets and yet GFP protein is only present from the DN3 subset onwards. This would suggest that the 5'UTR present in the proximal promoter cassette is still sufficient to allow the normal mechanisms of control to occur. In line LG-2, however, GFP transcripts and protein are present from DN1 cells onwards, suggesting the 5'UTR present is not able to confer appropriate control over downstream sequences. Thus, depending on the site of integration the 5'UTR sequences present in the proximal promoter expression cassette may have partial activity. Referring to Table 4.1 the steady state levels of GFP mRNA in total thymocytes correlates with the average GFP fluorescence of these cells. For example, total thymocytes from line LG-1 have the highest steady state level of GFP mRNA and the highest GFP fluorescence. This implies that the 5'UTR of GFP transcripts does not result in translational masking, since the level of GFP mRNA directly correlates with the level of protein. This discussion is based on the assumption that the AUG-containing 5'UTR of transcripts is important for the expression pattern seen in developing thymocytes. The future experiments suggested in section 3.5.1 need to be performed before any conclusions can be made in this regard (see figure 3.12).

4.4.2. The effect of integration site on transgene expression

The results presented here show that the steady state level of GFP mRNA in transgenic thymocytes is not directly proportional to the RCN of the founder line (Figure 4.10). This suggests that the p56\textsuperscript{Lck} proximal promoter sequences present within the expression cassette used here do not include sequences with LCR activity. Previous work analysing the expression of the full-length murine
genomic p56\textsuperscript{Lck} sequence under the control of the p56\textsuperscript{Lck} proximal promoter has reported the presence of LCR activity within this promoter when driving expression of the p56\textsuperscript{Lck} gene (Abraham et al., 1991a; Allen et al., 1992). The steady state level of transgenic mRNA was determined by the RCN of the transgenic line. However, in agreement with the data presented here, LCR activity was not detected when this promoter was used to drive the expression of heterologous genes. The authors postulated that sequence elements within the genomic sequence might be necessary for LCR activity of this promoter.

Due to the lack of evidence of LCR activity in the p56\textsuperscript{Lck} proximal promoter it is possible that the integration site of the transgene may effect its expression. Cytological studies of chromosomes, via techniques such as Giemsa staining, have revealed that chromatin exists in two major states. Heterochromatin is a densely staining form of chromatin that is highly condensed throughout the cell cycle. Euchromatin does not stain with Giemsa and appears to be decondensed during interphase. The chromatin structure of a gene locus can have powerful effects on whether the gene is expressed or not. Studies in Drosophila and yeast have shown that the expression of genes within, or adjacent to, regions of heterochromatin are effected by the highly condensed, compacted nature of the chromatin (for review see Karpen 1994). Chromosomes possess regions of constitutive heterochromatin, such as the centromere and telomere, next to junctional regions that have a random level of heterochromatin. Heterochromatin has been described as spreading along the chromosome resulting in heterochromatinization of juxtaposed euchromatic regions. This results in decreased accessibility of the transcriptional machinery to the genes in these regions, resulting in silencing of gene expression in a proportion of cells where they would normally be expressed. This form of silencing is termed position effect variegation (PEV) (for review see Karpen 1994). The expression of transgenes downstream of promoters with incomplete or absent LCR activity that integrate in junctional regions between heterochromatin and euchromatin can be randomly silenced in a proportion of cells lineage (Festenstein et al., 1996; Milot et al., 1996; Boyer et al., 1997). The degree of PEV is inversely proportional to the distance of the transgene from the
centre of the region of heterochromatin; the closer the transgene is placed to the
centromere or telomere; the higher the degree of variegation (Renauld et al.,
1993; Boyer et al., 1997). Expression cassettes that include an LCR, for example
the human CD2 cassette, have been shown to confer copy number dependent,
position independent transgene expression (Kioussis and Festenstein 1997).
LCRs may act by establishing, and/or maintaining, a stable open chromatin
structure of a locus, thus ensuring that regardless of the normal degree of
chromatin condensation at the site of integration, transcription factors can access
the necessary regulatory elements and direct transgene expression. Due to the
apparent lack of LCR activity within the p56Lck proximal promoter, the variable
patterns of GFP expression may, in part, be explained by PEV. In addition to
PEV, other position effects can impinge on transgene expression. Transgenes
can be affected by regulatory elements at the site of integration, which can
enhance or repress transgene expression. These additional position effects could
result in a change in the level of expression of the transgene within every cell,
rather than the on/off phenomenon caused by PEV.

In this study, two founder lines (LG-1 and LG-4) harboured transgenes
that had integrated relatively close to the centromere and two lines (LG-2 and
LG-3) harboured transgenes that have integrated close to the telomere (Figure
4.12A and B). Of the four lines analysed, line LG-4 showed the lowest
percentage of cells expressing GFP (Figures 4.7 and 4.8). Considering the close
proximity of this transgene to the centromere, the low percentage of GFP
expressing cells may result from PEV silencing of transgene expression within
these cells. The level of GFP mRNA expression in total thymocytes, as detected
by Northern blotting, was also the lowest in this line (Table 4.1). This technique
measures the average level of GFP mRNA in all thymocytes and does not
distinguish between GFP expressing and non-expressing cells. Such assays are
commonly used to assess transgene expression in tissue extracts. These
experiments do not allow effects such as PEV, which result in variegated
expression of the transgene, to be distinguished from other effects which result
in a decreased overall level of transgene transcription on a per cell basis.
Therefore, variegated expression of the transgene in line LG-4 may result in the
low percentage of GFP expressing cells. The low overall level of GFP expression, as detected by Northern blotting of total thymocytes, may result from a combination of PEV and additional PEV-independent, site of integration-mediated effects on the expression of the transgene.

In line LG-1 the transgene is also located in the pericentromeric region. The expression pattern of this transgenic line most accurately recapitulates the endogenous expression of p56\textsuperscript{Lck} protein, suggesting that position effects on the expression of the pLckGFP transgene is this line are minimal. However, the endogenous pattern of expression, where greater than 90% of DN3 and DN4 cells but less than 2% of DN1 and DN2 thymocytes express p56\textsuperscript{Lck} protein, was not completely reproduced. Only 46% of DN3 cells and 64% of DN4 cells are positive for GFP in this line. This could be due to PEV, resulting in silencing of expression of the transgene in a proportion of the DN3 and DN4 cells. In the DP and SP thymocytes GFP is expressed in over 90% of cells, as seen for endogenous p56\textsuperscript{Lck} protein. Why transgene expression in DP and SP thymocytes is less affected by PEV than it is in earlier DN thymocytes is unknown. Previous work in Drosophila has indicated that heterochromatin-mediated silencing of gene expression, although widespread in precursor cells, can be relaxed in terminally differentiated cells (Lu et al., 1996). A similar phenomenon has been described in transgenic mice utilising the human CD2 promoter linked to a disabled LCR (Festenstein et al., 1999). In one line of these mice a high level of variegation of hCD2 expression was seen in thymocytes but not in mature T cells. With respect to the expression of the pLck-GFP transgene, factors may be present in DP and SP thymocytes, that are absent from earlier thymocytes subsets, that can bind to the p56\textsuperscript{Lck} proximal promoter and contribute to the prevention of PEV. Studies aiming to elucidate the mechanism of LCR function have identified proteins that bind to LCRs and may regulate their function. For example, HMG box containing protein-1 (HBP1) binds to the human CD2 LCR (Zhuma et al., 1999). Deletion of the HBP1 binding site from an otherwise complete LCR results in variegation of transgene expression when the transgene is integrated close to regions of heterochromatin, suggesting an important role for this protein in LCR function. A similar role has been suggested for the LEF-1
HMG protein in the regulation of the human adenosine deaminase (ADA) LCR (Haynes et al., 1996). Thus, LCR binding proteins, with a role in establishing and maintaining an open chromatin configuration, may be present in DP and SP cells contributing to the absence of variegated expression of the pLck-GFP transgene at later stages of thymocyte development.

The two lines of pLckGFP transgenic mice which harboured transgenes at the telomeric end of the chromosome (lines LG-2 and LG-3) show distinct patterns of transgene expression. In line LG-2, over 80% of each DN thymocyte subset, as well as DP and SP thymocytes, expressed GFP (Figures 4.7 and 4.8). This transgene therefore does not appear to be influenced by PEV, since very little transgene silencing is seen. It is likely that this transgene is located in an open region of euchromatin where the transgenic DNA is easily accessible by transcription machinery, leading to constant gene expression. In contrast, in line LG-3 the transgene is expressed by a far lower percentage of cells. Less than 50% of DP thymocytes from this line express GFP, resulting in a bimodal distribution of cells either positive or negative for GFP. Such a distribution of transgene expression is characteristic of PEV. In this line the transgene has integrated very close to the telomere (region H3-4), possibly within telomeric sequences. Variegated expression of pericentromeric localised transgenes is well documented. Telomere-induced PEV has also been described in yeast and in mice (Gottschling et al., 1990; Renauld et al., 1993; Zhumaa et al., 1999). To further analyse the expression pattern of GFP from this locus homozygous LG-3 mice were generated. As described the homozygous mice expressed GFP in a higher percentage of cells (Figure 4.13B). In the heterozygous line LG-3 mice 52% of total thymocytes expressed GFP. If transgene activation is random, as expected for PEV (Elliott et al., 1995) and the individual loci in a homozygous cell are stochastically activated or silenced then in a homozygous cell the probability of expressing GFP should increase from 50% to 75%. Two thirds of these cells should have one locus active and approximately a third should have both transgene loci active. As shown in Figure 4.13B, 83% of DP cells from the homozygous line are GFP positive. These results are what would be expected from a stochastic activation of the GFP transgene, as may occur during PEV. The
increase in overall GFP fluorescence in this homozygous line may reflect the inclusion of cells expressing the transgene from both chromosomes. One possible explanation for line LG-3, but not line LG-2, being influenced by PEV is the different RCNs of these lines (RCN of 20 and 4 respectively). The degree of PEV in Drosophila has been shown to increases proportionally with the copy number of repeats present at the transgenic locus (Dorer and Henikoff 1994). Similar copy number dependent silencing has also been described in plants (Flavell 1994). In mice, the influence of RCN on PEV is less clear. Some studies do show an increased degree of variegation with higher copy number transgenic mice (Garrick et al., 1996; Robertson et al., 1996) but other studies see no correlation between these two factors (Zhuma et al., 1999).

Due to the apparent lack of LCR activity in the p56Lcκ proximal promoter cassette used here, it is conceivable that position effects due to chromosomal integration site may be at least partly responsible for the variability of GFP expression. The potential, and somewhat complex, involvement of PEV and other site-mediated effects on transgene expression for each founder line has been discussed. Taken together, these data demonstrate that the expression of at least one transgene product under the control of the proximal p56Lcκ promoter can vary between transgenic lines and does not always faithfully recapitulate the expression of endogenous p56Lcκ mRNA or protein. This study underscores the importance of the analysis of multiple transgenic founder lines when using transgenic mice to investigate the consequences of tissue specific expression of a gene. Due to the potential influence of chromosomal integration site on transgene expression when using the p56Lcκ proximal promoter these data also indicate that it can be important to analyse mice in which the transgene has integrated into an open area of chromosome. In addition, the assumption that the transgenic line with the highest RCN will express p56Lcκ proximal promoter driven transgenes at the highest level should not be made. If possible the expression of such transgenes should be analysed with assays that can distinguish individual cells to check for variegated expression. Previous studies expressing dominant negative transgenes under the control of the p56Lcκ proximal promoter to elucidate the role of an endogenous gene in thymocyte
development may have underestimated the importance of these genes. Incomplete blocks in thymocyte development following the expression of such transgenes may result from effects such as PEV resulting in a proportion of thymocytes failing to express the transgene and so developing normally. These results, therefore, have implications for the use of the p56\textsuperscript{Lck} proximal promoter in the generation of transgenic mice and also for the interpretation of the phenotypes of previously generated transgenic animals that utilised the p56\textsuperscript{Lck} proximal promoter.
Chapter 5
Generation and analysis of CBF dominant negative transgenic mice

5.1. Introduction

Haematopoiesis is a lifelong process in which pluripotent, self-renewing stem cells gives rise to all blood cell lineages. These processes are controlled by transcription of lineage-specific genes that encode for proteins allowing for cellular proliferation, survival and differentiation. Many transcription factors are required for haematopoiesis, as demonstrated by analyses of mice deficient for transcription factor genes and from the characterisation of chromosomal rearrangements in human leukaemia. The core binding factor (CBF) family of transcription factors, also known as the polyoma virus enhancer-binding protein 2 (PEBP2) or the acute myeloid leukaemia protein (AML) family, are known to be essential for normal haematopoiesis. The work presented here aims to elucidate the role of this family in thymocyte development.

5.1.1. Identification of the CBF family

The CBF family of heterodimeric transcription factors are comprised of two unrelated subunits, a DNA binding α subunit and a non DNA-binding β subunit (Kamachi et al., 1990; Wang and Speck 1992; Ogawa et al., 1993b; Wang et al., 1993). The mammalian CBFα subunits are encoded by three distinct genes CBFα1/PEBP2αA/AML3 , CBFα2/PEBP2αB/AML1 and CBFα3/PEBP2αC/AML2 while the common β subunit is encoded by the CBFβ gene (Bae et al., 1993; Ogawa et al., 1993b; Levanon et al., 1994; Bae et al., 1995). PEBP2 was originally identified as a factor that only becomes detectable in F9 embryonal carcinoma cells after they are induced to differentiate (Krysze et al., 1987; Furukawa et al., 1990). Since the polyoma virus enhancer is inactive in F9 cells until they differentiate, PEBP2 was thought to be involved in control of the differentiation-dependent activity of this enhancer. PEBP2 specifically recognises the enhancer core sequence TGYGGT which was first identified in the polyoma virus
enhancer (Kryszke et al., 1987; Kamachi et al., 1990). Independently CBF was identified as a factor binding to the same core sequence in the murine leukaemia virus enhancer and was found to be identical to PEPB2 (Wang and Speck 1992). The PEBP2/CFB family of transcription factors have since been found to represent murine homologues of a human gene AML which also encodes a RUNT-domain containing protein (Miyoshi et al., 1991; Erickson et al., 1992). CBFα1 is also known as OSF2 (osteoblast-specific transcription factors 2) following identification as a factor binding to the osteocalcin gene promoter (Ducy et al., 1997). Due to the presence of multiple names in current use for each of the three genes encoding the α subunits it been suggested that the CBFα family be renamed. The gene name RUNX has been suggested but is yet to be accepted; within this thesis the genes will be termed CBFα 1, 2 and 3 as designated above (for review see Ito 1999).

The CBFα subunits contain a conserved 128 amino acid DNA binding domain, known as the RUNT domain because of its high homology to the Drosophila pair-rule gene runt (Kania et al., 1990). The runt gene encodes for a protein which has a vital role during Drosophila embryogenesis in the formation of the segmented body pattern and also functions in the nervous system and in sex determination (Duffy and Gergen 1991; Duffy et al., 1991). It is through the RUNT sequence that the α subunit binds to DNA and interacts with other proteins including the β subunit (Kagoshima et al., 1993; Meyers et al., 1993; Ogawa et al., 1993b; Kagoshima et al., 1996). Although the β subunit does not bind to DNA itself it is important for the stabilisation of DNA binding by the α subunit (Ogawa et al., 1993a; Wang et al., 1993). Initial analysis revealed that the α subunit can bind DNA without the β subunit but with a reduced efficiency (Ogawa et al., 1993a) but later work revealed that the CBFβ subunit is essential for CBFα2 to function in vivo (Wang et al., 1996c).

5.1.2. The link between CBF and leukaemia

The CBFα2 and CBFβ genes have been found to be frequently disrupted in human leukaemia. Together translocations and inversions involving these two genes are associated with up to 30% of de novo cases of acute myeloid leukaemia (AML) and up to 28% of paediatric acute lymphoblastic leukaemia (ALL) (for reviews see Nucifora and Rowley 1994; Look 1997). The CBFα2 gene,
located on chromosome 21, band q22, was first identified at the breakpoint of the t(8;21) translocation characteristic of AML (Miyoshi et al., 1991). This chromosomal aberration is among the most commonly found in the M2 type AML (Rowley 1990). *CBFa2* is also disrupted by other translocations, for example t(3;21) in therapy related leukaemia (Nucifora et al., 1993a; Mitani et al., 1994) and t(12;21) in AML and ALL (Golub et al., 1995). These translocations create chimeric proteins containing peptide domains of the *CBFa2* protein, including its DNA-binding domain, fused to sequences from other proteins. These proteins include ETO in the case of t(8;21) (Erickson et al., 1992), EAP/EVI1/MDS1 in t(3;21) (Nucifora et al., 1993a; Nucifora et al., 1993b; Mitani et al., 1994) and TEL in the t(12;21) translocation (Golub et al., 1995). The *CBFB* subunit is also disrupted by inversion inv(16) and t(16;16) in AML (Liu et al., 1993; Hajra et al., 1995). The chimeric proteins resulting from the inv(16) and t(16;16) contain the heterodimerization domain in *CBFB* fused to the coiled-coil tail region of a smooth muscle heavy chain (Liu et al., 1993; Hajra et al., 1995). The mechanism by which the chimeric proteins generated by the chromosome alterations of the *CBFa2* and *CBFB* genes result in leukaemogenesis are not fully understood. The most widely accepted theory is that these proteins mediate their affects via interfering with the normal function of CBF. A study of patients with familial platelet disorder who are defective in platelet production and are predisposed to AML confirmed this theory (Song et al., 1999). Mutational analysis of these patients revealed the presence of mutations or intragenic deletion of one allele of the *CBFa2* gene. Haploinsufficiency of *CBFa2* can therefore result in predisposition to AML, indicating that interference with *CBFa2* normal function can lead to leukaemia. Therefore, the *CBFa2* and *CBFB* genes are both independently targeted by chromosomal translocations that can contribute to the development of leukaemia.

5.1.3. **The CBF transcription factors are important regulators of T cell specific gene expression.**

The CBF α/β heterodimeric protein complex binds the enhancer core sequence TGYGGGT which was originally identified in the polyoma virus enhancer and murine leukaemia virus enhancer (Kamachi et al., 1990; Wang and Speck 1992). This sequence motif is also present in the enhancers of a variety of
T cell specific genes, including those that encode the TCRα chain and the TCRβ chain (Krimpenfort et al., 1988; Ho et al., 1989; Gottschalk and Leiden 1990; Ogawa et al., 1993b), the TCRγ chain and the TCRδ chain (Redondo et al., 1992; Wang and Speck 1992; Hsiang et al., 1993), CD2 (Lake et al., 1990) and CD3ε (Hallberg et al., 1992). A role for CBF transcriptional regulation in T cells has been analysed by mutational analysis of the minimal T-cell specific enhancer fragments from the TCRα or β genes. The minimal human TCRβ gene enhancer contains two phorbol ester-inducible elements (Tβ3 and Tβ4) which each include the core binding sequence. Mutation of these sites leads to a severe reduction in enhancer activity, revealing a crucial role for CBF in the regulation of TCRβ gene expression (Ogawa et al., 1993b). Furthermore, another report revealed that mutations within the TCRδ enhancer, falling within its CBF binding site, resulted in a reduction in its enhancer activity (Redondo et al., 1992).

CBF binding sites are frequently adjacent to binding sites for other transcription factors, such as c-myb, Ets-1 and AP-1, in enhancer sequences. CBF has been shown to co-operate with these factors to facilitate transcriptional activation. For example, the enhancer sequences within the long terminal repeats (LTRs) of the murine leukaemia virus SL3-3 contains CBF sites flanking binding sites for c-myb and Ets-1 (Zaiman and Lenz 1996). Transient transfection assays revealed that c-myb and CBF co-operatively stimulate transcription from the LTR (Zaiman and Lenz 1996). Similarly CBF and c-myb co-operate for the T cell specific expression of TCRδ (Hernandez-Munain and Krangel 1994). In the TCRβ enhancer, CBF binding sites in the Tβ3 and Tβ4 regions are located adjacent to binding sites for Ets-1. Mutagenesis studies have revealed that the mutation of either binding site reduces enhancer activity and that the Ets and CBF factors bind co-operatively (Wotton et al., 1994). In addition, several transcription factors, including LEF-1, CREB, Ets-1 and CBF, bind to the minimal TCRα enhancer. Mutations in any one of these sites result in severe reduction in enhancer activity (Giese et al., 1995). Again co-operativity was shown between the binding of CBF and Ets-1 (Giese et al., 1995). Therefore, through multiple protein-protein interactions and the formation of higher-order nucleoprotein complexes, CBF is necessary for transcriptional activation of the
above mentioned T cell specific genes. CBF binding sites are also present within the promoters of numerous other genes and have been shown to be important for the regulation of expression of these genes whose products are important for haematopoiesis and bone formation (see Table 5.1)

Table 5.1: A summary of CBF binding sites present within promoters of non-T cell specific genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Other details of gene product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>interleukin 3 (IL-3)</td>
<td>Haematopoietic cell specific cytokine</td>
<td>(Cameron et al., 1994; Uchida et al., 1997)</td>
</tr>
<tr>
<td>colony-stimulating factor receptor (CSF-1)</td>
<td>Receptor for a monocyte specific cytokine</td>
<td>(Zhang et al., 1994)</td>
</tr>
<tr>
<td>granulocyte macrophage colony stimulating factor (GM-CSF)</td>
<td>Haematopoietic cell specific cytokine</td>
<td>(Takahashi et al., 1995; Cockerill et al., 1996)</td>
</tr>
<tr>
<td>Myeloperoxidase (MPO)</td>
<td>Antimicrobial myeloid cell specific enzyme</td>
<td>(Nuchprayoon et al., 1994)</td>
</tr>
<tr>
<td>neutrophil elastase</td>
<td>Antimicrobial myeloid cell specific enzyme</td>
<td>(Suzow and Friedman 1993)</td>
</tr>
<tr>
<td>granzyme B serine protease</td>
<td>Cytotoxic protease induced in activated T cells</td>
<td>(Wargnier et al., 1995)</td>
</tr>
<tr>
<td>defensin protein NP3</td>
<td>Antimicrobial cytotoxic neutrophil protein</td>
<td>(Westendorf et al., 1998)</td>
</tr>
<tr>
<td>osteocalcin</td>
<td>Osteoblast and odontoblast specific enzyme</td>
<td>(Ducy and Karsenty 1995; Geoffroy et al., 1995; Frendo et al., 1998)</td>
</tr>
<tr>
<td>osteopontin</td>
<td>Bone matrix protein, expressed in various cell types</td>
<td>(Sato et al., 1998; Jimenez et al., 1999)</td>
</tr>
<tr>
<td>collagenase 3</td>
<td>Matrix metalloproteinase, degrades collagen. Expressed by chondrocytes</td>
<td>(Pendas et al., 1997; Tardif et al., 1997),</td>
</tr>
<tr>
<td>bone sialoprotein (BSP)</td>
<td>Bone specific extracellular matrix protein</td>
<td>(Yang and Gerstenfeld 1997)</td>
</tr>
<tr>
<td>transforming growth factor (TGFβ type 1 receptor)</td>
<td>Receptor for TGF-β, enhances bone matrix synthesis and repair</td>
<td>(Chang et al., 1998; Ji et al., 1998)</td>
</tr>
</tbody>
</table>

5.1.4. CBFα1 and CBFα2 are expressed in T cells throughout their development

CBFβ mRNA has been detected in a broad range of mouse cell lines including fibroblasts, T cells and B cells (Ogawa et al., 1993a). It is also expressed in various tissues including brain, lung, heart and thymus and its expression is thought to be ubiquitous (Satake et al., 1995). This is in contrast to the varying levels of CBFα that have been detected in different cell types. CBFα mRNA is detectable in Ha-ras-transformed NIH 3T3 cells, undetectable in undifferentiated F9 cells (Furukawa et al., 1990) and is preferentially expressed in T cell lines as
compared to B cell lines (Ogawa et al., 1993b). These investigations did not however distinguish between the different CBFα isoforms. Further data indicate that the CBFα genes have a specific tissue expression pattern. The expression of CBFα3 mRNA within the thymus is as yet undetermined but this isoform is present in various haematopoietic cell lines, including HL60 myeloid, Jurkat T and Daudi B cell lines (Levanon et al., 1994). Both CBFα1 and CBFα2 have been detected within the thymus. CBFα1 mRNA has been found in T cell lines, NIH 3T3 cells, thymus and testes but not in other tissues including brain, lung, heart, spleen or liver (Satake et al., 1995). Recent work has demonstrated that CBFα1 mRNA is expressed in bone at a twenty-times higher level than in thymus (Banerjee et al., 1997; Ducy et al., 1997). CBFα2 mRNA is expressed in most haematopoietic cell lines and may also be expressed in myeloblasts or erythroblasts (Miyoshi et al., 1991; Bae et al., 1993; Levanon et al., 1994). It has been detected in thymus and at lower levels in testes, lung and heart (Satake et al., 1995). In addition, CBFα2 mRNA is expressed in skeletal muscle (Zhu et al., 1994). Within the thymus CBFα1 and CBFα2 are expressed in day 16 embryos, new born and 4 week old mice. These two CBF isoforms are also expressed throughout T cell development, in DN, DP and SP populations (Satake et al., 1995). The expression of CBFα1 and CBFα2 in T cells throughout their development supports the notion that the CBF family of transcription factors play a role in T cell differentiation.

5.1.5. Biological functions of the CBF family

5.1.5A. A role for CBFα1 in bone development

CBFs play critical roles both in normal developmental processes and in disease. The Cbfα1 gene, encoding for the Cbfα1 subunit, is required for normal bone development. Two types of bone formation have been identified within mammalian embryogenesis. The majority of bones are laid down as a framework or anlage of hyaline cartilage in a process termed endochondral ossification. Subsequent intramembranous ossification then occurs within this framework. The second type of bone development, termed intramembranous ossification, occurs without a cartilage framework and generates such bones as the flat bones of the skull and the clavicles (see Erlebacher et al., 1995 for
Targeted disruption of CBFα1 in mice resulted in the absence of mature osteoblasts (bone forming cells) and a complete lack ossification, both intramembranous and endochondral (Komori et al., 1997; Otto et al., 1997). Immature osteoblasts do appear but these are maturationally arrested, lacking osteopontin and osteocalcin expression and expressing only low levels of alkaline phosphatase (Komori et al., 1997). CBFα1 deficient mice die of respiratory failure shortly after birth presumably due to their soft cartilaginous ribcage providing inadequate support for respiration. The CBFα1 gene has been mapped to chromosome 6p21 which is also the locus for an autosomal dominant human bone disease, cleidocranial dysplasia (CCD) (Levanon et al., 1994; Mundlos et al., 1995). Heterozygous patients with CCD have defects in both endochondral and intramembranous bone formation. They are typically of short stature and present with certain skeletal abnormalities such as hypoplastic/aplastic clavicles, patent anterior fontanelles and supernumerary teeth. Interestingly the skeletal changes seen in the heterozygous CBFα1 deficient mice are similar to those seen in CCD (Otto et al., 1997). Subsequent work has confirmed the presence of CBFα1 mutations in patients with CCD (Lee et al., 1997; Mundlos et al., 1997; Zhang et al., 2000).

5.1.5B. A role for CBF in haematopoietic development

Mutations in several genes encoding transcription factors, such as Pu-1 and Ikaros, have profound effects on haematopoiesis. Gene disruption studies have allowed the role of CBF in haematopoietic development to be investigated. CBFα2 deficient mice show normal morphogenesis and yolk-sac-derived primitive erythropoiesis; however they lack definitive myeloid or erythroid progenitors in both the yolk sac and liver (Okuda et al., 1996; Wang et al., 1996b). These mice exhibit failure of foetal liver haematopoiesis and die between embryonic day 11.5 and 12.5 due to haemorrhaging in the central nervous system (CNS). In addition, CBFα2 deficient ES cells failed to contribute to haematopoiesis in chimeric animals (Okuda et al., 1996). Therefore, CBFα2 and the target genes it regulates are essential for definitive haematopoiesis of all lineages. Mice deficient for CBFβ have also been generated and they display a very similar phenotype to the Cbfα2 deficient mice (Sasaki et al., 1996; Niki et al.,
Homozygous CBFβ mutant mice die at around embryonic day 12.5, again due to haemorrhaging in the CNS. These mice exhibited primitive erythropoiesis in the yolk sac but lacked definitive haematopoiesis in foetal liver. As discussed, CBFα1 deficient mice fail to develop mature osteoblasts and lack ossification (Komori et al., 1997; Otto et al., 1997). The only non-skeletal defect observed in these mice was an increased level of primitive nucleated erythrocytes. The authors speculate that this is a secondary effect of the lack of haematopoietic bone marrow development. Haematopoiesis was normal in these mice, suggesting that CBFα1 does not play an essential role in these processes. These data underscore the importance of CBFα2 and CBFβ in definitive haematopoiesis. This essential role for CBF in haematopoiesis was predicted from the previously mentioned involvement of CBFα2 and CBFβ genes in various forms of human leukaemia.

5.1.6. Generation of CBF dominant negative transgenic mice to investigate the role of the Core Binding Factors in thymocyte development

The critical role for members of the CBF family in bone development and haematopoiesis has been described. The importance of these transcription factors in thymocyte development is yet to be elucidated. The CBFα genes are expressed throughout thymocyte development, implicating a role for the CBF family in thymocyte differentiation. In addition, binding of the heterodimeric αβ CBF complex to gene enhancers has been shown to be required for the transcriptional activation of numerous T cell specific genes, including those that encode for chains of the TCR. Interference with CBF activity may therefore result in thymocyte development being arrested at the double negative CD44-CD25+ (DN3) thymocyte stage due to cells failing to express a TCR. A number of approaches could be taken to investigate the role of CBF in these processes. The early lethality of the CBFα2 and CBFβ deficient mice precludes the study of the role of CBF proteins in T cell development and function in these mice. Thymocyte development in the CBFα2 and CBFβ heterozygous mice has not been documented and these mice were unavailable when this project was initiated. The haematopoietic defect in the CBFα2 deficient mice appears to be intrinsic to haematopoietic stem cells or multipotent progenitors since CBFα2 deficient ES cells failed to contribute to haematopoiesis in Rag-chimera.
experiments (Okuda et al., 1996). This technology can therefore not be utilised here. Thymocyte development was normal in the CBFα1 deficient mice, suggesting that other family members are involved or that the role of CBFα1 in thymopoiesis is redundant. To investigate the role of the heterodimeric α/β CBF complex in thymocyte differentiation, transgenic mice expressing a dominant negative form of CBF within developing thymocytes will be generated here.

There are reasons to believe that CBFα/β levels are limiting, therefore the expression of low levels of a competing protein should effectively reduce the level of functional CBF within these cells and allow its role in thymocyte development to be analysed (Wang et al., 1996c). CBFα2 deficient mice lack definitive haematopoiesis and fail to develop any erythroid or myeloid progenitors. Mice that are heterozygous for CBFα2 develop fewer of these progenitors than wild-type mice do. This indicates that the level of CBFα/β in haematopoietic cells is limiting and that a reduction of this level results in a block in haematopoiesis. The dominant negative constructs used in these experiments are the DNA binding Runt domain of CBFα, expressed under the control of the p56^{leuk} proximal promoter. It is through the Runt sequence that the α subunit binds to DNA (Kagoshima et al., 1993; Meyers et al., 1993; Ogawa et al., 1993b; Kagoshima et al., 1996). The transgenic protein, consisting of the DNA binding domain alone without the downstream transactivation domain, should compete with endogenous CBFα/β complexes for DNA binding sites. Previous work has demonstrated that expression of a truncated RUNT region alone can compete with DNA binding of full-length CBFα2 (Sakakura et al., 1994). This transgenic protein should interfere with CBF function since it has been shown that the DNA-binding domain of runt-related genes have no transactivation function on their own (Bae et al., 1994; Ducy and Karsenty 1995). Since the Runt domain is common to all of the CBFα proteins expression of this construct should target all three CBFα proteins and so overcome functional redundancy between these family members. In addition, a second construct with a mutated RUNT sequence will be generated. The point mutation within this RUNT sequence was first identified in a patient with CCD and has been shown to abolish its binding to DNA (Lee et al., 1997). The transgenic protein produced
by this construct should be unable to bind to DNA, so transgenic mice generated with this construct should act as controls.

For the CBF/RUNT protein to act in a dominant negative fashion it must be expressed within the cell at the same location as the endogenous CBF protein. Immunofluorescence labelling of NIH 3T3 cells transfected with cDNAs for CBFα and CBFβ demonstrated that CBFα is a nuclear protein and that CBFβ is cytoplasmic (Lu et al., 1995). The CBFα and CBFβ subunits are thought to heterodimerize within the cytoplasm and translocate to the nucleus to regulate transcription of downstream target genes. It is therefore essential that the transgenic protein will also be targeted to the nucleus. Two regions of CBFα are responsible for the nuclear accumulation of the protein (Lu et al., 1995). One element was identified in the carboxyl terminus (between amino acids 221 and 513) and the other mapped to the RUNT region. Complete removal of the RUNT domain resulted in the majority of protein staying in the cytoplasm. When the 221-513 region was deleted, although some protein was observed in the cytoplasm, the majority of fluorescence was detected in the nucleus. These results indicate that the RUNT sequence contains a nuclear localisation signal (NLS) strong enough to ensure that the majority of the transgenic RUNT protein will be targeted to the nucleus where it can function.

For the dominant negative molecule to interfere with the function of the CBFα/β complex it needs to be expressed at the appropriate time within thymocyte development. As previously described, CBF is required for the efficient activity of the TCRβ enhancer (Ogawa et al., 1993b). It is therefore important that the dominant negative CBF/RUNT protein is available to compete with endogenous CBF at the time when this enhancer is normally active. The p56Lck proximal promoter is commonly used to express transgenes within developing thymocytes. From the generation of pLck-GFP transgenic mice we have gained a number of insights into the use of this promoter (Chapter 4). Despite being subject to position effects relating to the site of transgene integration the pLck-GFP transgenes were consistently expressed in DN3 thymocytes and even as early as the DN1 population depending on the founder
line analysed (Figure 4.8). Therefore, the p56\textsuperscript{Lck} proximal promoter will be used to drive the expression of the CBF/RUNT transgenes within developing thymocytes in an attempt to elucidate the role of the CBF\textgreek{a/\beta} transcription factors in T cell differentiation.

5.2. Specific Methods

5.2.1 Transgenic Constructs design

5.2.1A. pLck-RUNT-Engrailed constructs
The murine CBF\textgreek{a} cDNA was PCR amplified from reverse transcribed total thymus RNA. From this PCR product the 396 base pair Nco1-HindIII fragment encoding the DNA-binding domain RUNT domain was amplified using the primers RUNT 1 and RUNT 2 (see below). The 396 base pair Nco1-HindIII fragment was also used as a RUNT-specific probe for the detection of RUNT-containing transcripts on Northern blotting analysis. A non-binding RUNT sequence was amplified from the pCMV-hOSF2\textgreek{a}-MET construct (gift from Dr G Karsenty) using primers MUT-RUNT 1 and RUNT2 (see below). The RUNT sequence within this construct contains a methionine mutation (Met175Arg) that has been shown to abolish binding of the RUNT domain (Lee et al., 1997). (Note the RUNT 1 primer and the MUT-RUNT 1 primers both include sequences for a translational start site). The RUNT sequences were cloned independently into the pSKM\textgreek{e}n plasmid. This construct contains an eleven amino acid myc tag for identification purposes and the repressor domain (amino acids 2 to 298) of the \textit{Drosophila} Engrailed (En) transcription factor. The RUNT-containing PCR products were digested with Xho1, end-filled with Klenow and blunt ligated into the pSKM\textgreek{e}n plasmid (previously digested with Xho1, endfilled with Klenow and dephosphorylated with calf intestinal phosphatase (CIP)). The WT-RUNT-myc-Engrailed and the MUT-RUNT-myc-Engrailed sequences, both including a 3’ STOP codon, were excised from the pSKM\textgreek{e}N construct by digestion with BssH1. These cDNA s were endfilled with Klenow and cloned into the p56\textsuperscript{Lck} proximal promoter expression cassette (previously digested with BamH1, endfilled with Klenow and dephosphorylated with CIP). These constructs are hereafter referred to as pLck-WT-RUNT-Engrailed and pLck-
MUT-RUNT-Engrailed (see Figure 5.2). The WT-RUNT-myc-Engrailed sequences and MUT-RUNT-myc-Engrailed sequences were also cloned into pBluescript (pBS-WT-RUNT-Engrailed and pBS-MUT-RUNT-Engrailed).

RUNT 1: 5′ – cgccctcggagatccggcatggtggagatccg-3′
RUNT 2: 5′ – gccctcggagaagcttctgtctgtgccttc-3′
MUT RUNT 1: 5′ – cgccctcggagatccggcatggtggatcgcc-3′

5.2.1B. pLck-Flag-RUNT constructs
As described above, sequences for the wild type RUNT domain were amplified from the murine CBFα1 cDNA and sequences for the non-binding MET-mutant RUNT were amplified from the pCMV-hOSF2α-MET construct using primers RUNT-3 and RUNT-4 (see below). The RUNT-3 primer includes sequences for a translational start site and for a Flag tag. Cloning of this Flag coding sequence adjacent to the RUNT coding sequence results in fusion of the Flag marker peptide to the RUNT protein. The Flag peptide can be recognised by the anti-Flag M2 antibody, allowing cells expressing the transgenic protein to be identified (Kodak). RUNT-4 primer includes sequences to ensure that the CBFα1 nuclear localisation signal (NLS) is maintained (Thirunavukkarasu et al., 1998) and a stop codon. The Flag-WT-RUNT and Flag-MUT-RUNT sequences were digested with BamH1 and cloned into the BamH1 cloning site of the p56Lck proximal promoter expression cassette to generate pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT respectively (Figure 5.4). In addition, these sequences were cloned into pBluescript and pCDNA3 generating pBS-Flag-WT-RUNT, pBS-Flag-MUT-RUNT, pCDNA3-Flag-WT-RUNT and pCDNA3-Flag-MUT-RUNT.

RUNT 3: 5′ -cgc gga tcc gcc atg gac tac aag gac tac aag gac gat gac aag gac tac aag gac gat gac aag gac cac-3′
RUNT 4: 5′ –gcc gga tcc tca gtc agt cat caa gct tgt gcc ttc tgt gtt ccc ggg g-3′
5.2.2. Typing of pLck-Flag-RUNT transgenic mice via PCR

The primers shown below (RUNT Tg1 and RUNT Tg2) were used. RUNT Tg1 is specific for sequences within the p56\textsuperscript{Lck} proximal promoter 5' of the cloning site and RUNT Tg2 is specific for sequences within the 3' end of the RUNT sequence. These primers generate a 550 base pair product.

RUNT Tg 1: 5' – gcg aca tgt gtt gtt gta tct ccc -3'
RUNT Tg 2: 5’ - cac tgt cac ttt aat agc tct gtg -3'

PCR conditions were as described in section 2.7. Cycles were as follows:
First cycle: denaturation at 94°C for 4 minutes
         annealing at 52°C for 30 sec
         extension at 72°C for 60 sec
Subsequent 39 cycles: denaturation at 94°C for 30 sec
         annealing at 52°C for 30 sec
         extension at 72°C for 60 sec
Final extension period: 72°C for 5 minutes

5.2.3. Preparation of nuclear/cytoplasmic protein extracts

Single cell suspensions of thymocytes were prepared from transgenic and non-transgenic mice (10\textsuperscript{6} total) and were washed twice with PBS, each time centrifuging the cells at 13000rpm at 4°C. The cell pellet was resuspended in 0.5ml Buffer A plus 0.15% NP40 and incubated on ice for 1 minute. The samples were then centrifuged for 1 minute. The supernatant, which contains cytoplasmic protein extracts, was transferred to a clean tube and precipitated on ice with 0.7ml acetone and stored at -70°C. The remaining pellet was washed twice in 0.5 Buffer A (without NP40) to remove any remaining cytoplasmic contaminants. The nuclei were then very gently resuspended in 0.5ml Buffer B. The sample was mixed by rotation at 4°C for 30 minutes before centrifugation at 4°C at 13000rpm for 20 minutes. The supernatant, containing nuclear proteins, was removed, precipitated on ice with 0.7ml acetone and stored at -70°C. 25μl of SDS loading buffer was added to each sample before separation by electrophoresis on an 8% SDS-polyacrylamide gel (SDS-PAGE). Primary
antibodies used were as follows; an anti-Flag mouse monoclonal (IgG1, Santa Cruz; sc1656) and an anti-STAT5 mouse monoclonal (IgG1, Santa Cruz, sc 7787). The secondary antibody was coupled with horseradish peroxidase and detected with ECL-kit (Amersham).

**Buffer A:**
- 10mM HEPES (pH 7.9), 15mM KCl, 2mM MgCl₂,
- 0.1mM EDTA pH 8.0, 10mM NaF
- 1mM DTT (added fresh), 1mMPMSF (added fresh)
- 1mM Na vanadate (added fresh)

**Buffer B**
- 20mM HEPES (pH 7.9), 20% glycerol, 0.42 M NaCl
- 1.5mM MgCl₂, 0.2mM NaF, 1mM DTT (added fresh)
- 1mM Na vanadate (added fresh),
- 1mMPMSF (added fresh)

### 5.2.4. In vitro translation of pLck-RUNT constructs

To translate cDNA sequences into protein *in vitro* the TnT T7 quick coupled reticulocyte lysate system was used (Promega). This system allows for the transcription and translation of genes cloned downstream from the T7 polymerase promoter. 0.2-2.0μg of the DNA to be translated and 1mM S³⁵ labelled methionine was added to a tube containing the master mix (includes the RNA polymerase, nucleotides, salts, RNAsin ribonuclease inhibitor and the reticulocyte lysate solution) and following incubation at 30°C for 90 minutes protein was produced. The proteins synthesised were then analysed by SDS-PAGE and autoradiography.

### 5.2.5. Transient transfections and Luciferase assays

#### 5.2.5A. Transient transfections

Human bladder carcinoma cells (line 5637) were cultured in DMEM medium containing 5% FCS (DMEM+5%FCS) in a humidified atmosphere of 10% CO₂ at 37°C. Transfections were carried out using calcium phosphate. 24 hours prior to transfection, exponentially growing cells were harvested via trypsinisation and replated at 0.5 x 10⁶ per 6cm plate, in a total of 4ml DMEM+5%FCS. 2 hours prior to transfection cells were washed with
DMEM+5%FCS. The calcium-phosphate-DNA coprecipitate was then prepared: the appropriate amounts of each plasmid, to a total of 12µg (kept constant with the addition of pBS plasmid), were added with 31µl 2M CaCl₂ and made up to 250µl with distilled water. For each assay 2µg n1-EGFP (Clontech) was included and GFP expression was used to normalise each assay for transfection efficiency. To study the effect of the Flag-WT-RUNT and Flag-MUT-RUNT proteins the pCDNA3-Flag-WT-RUNT and pCDNA3-Flag-MUT-RUNT constructs were used for these transfections. pCDNA3 is designed for high level constitutive expression in a variety of mammalian cell lines (Invitrogen). In each transfection the total quantity of pCDNA3 constructs was equivalent; i.e. made up to the same amount with vector-alone pCDNA3. The DNA mix was then added dropwise to the same volume of 2xHBS. After 20 minutes incubation at room temperature, during which time a fine precipitate formed, the suspension was added dropwise onto the cell monolayer. The plates were rocked gently from side to side before being placed back into the incubator. 24 hours later the cells were washed with PBS and 4ml of DMEM+5%FCS was added. After an additional 24 hours the cells were harvested via trypsinisation and lysates were prepared for luciferase assay transfection.

2xHBS 280 Mm NaCl, 10mM KCl, 12mM dextrose
1.5mM Na₂HPO₄.2H₂O, 50mM HEPES

5.2.5B. Luciferase assays

Luciferase assays were performed using the luciferase assay system following the manufacturers protocol (Promega). In brief, cells were washed with PBS before being lysed with 250µl of 1x reporter lysis buffer. Following rocking at room temperature for 10 minutes a rubber policeman was use to scrape the lysed cells off the plate and the lysate was collected into a clean Eppendorf. The lysate was vortexed for a few seconds and centrifuged at 13000 rpm for 15 seconds to pellet cell debris. The supernatant was then transferred into a clean Eppendorf tube and the lysate stored at -70°C. Following thawing 100µl of each lysate was aliquoted into individual wells of a 96 well plate and GFP readings for transfection efficiency were obtained using a fluorometer and Cytol-1 software. Luciferase readings were taken from the same plate on a luminometer via a flash assay using the Revelation software.
5.2.6. Thymocyte survival in vitro

Single cell suspensions of thymocytes were prepared as previously described. Cells were cultured in complete DMEM medium (DMEM-1640 plus 5% FCS, 50μM β-mercaptoethanol and penicillin/streptomycin) at 10^6 cells per ml in 24 well plates. Cells were either untreated or were treated with TGFβ1 (human platelets, Calbiochem) added to a final concentration of 1ng/ml, or Dexamethasone (Sigma) at a final concentration of 0.1μM, or were suspended in media without serum. The samples were then incubated at 37°C in a humidified atmosphere of 10% CO₂. At each time point cells were processed to determine the percentage of cells undergoing apoptosis. Results are representative of four independent experiments. All statistical analyses were performed by Generalised Estimating Equations (GEE) (kindly carried out by M Bradburn) in which the intra-mouse correlation in response is taken account of. An exponential decay model was assumed. A statistically significant result is evidence of a differential decay rate between the groups. The model was constrained to allow the survival at time zero to be 100%. For the analyses of sub G1, the analysis differed in that the response variable was the logarithm of (100-percentage in sub G1) and no constraint was imposed on the intercept.

5.2.6A. Annexin V Staining

Annexin V is a 35-36 kDa calcium dependent, phospholipid-binding protein that has high affinity for phospholipid phosphatidylserine (PS) residues that are externalized in cells that are in the early stages of apoptosis (Raynal and Pollard 1994). Staining with Annexin V in conjunction with Topro-3 iodide dye allows dead and apoptosing cells to be identified. Topro-3 iodide dye is impermeant to the membranes of live cells but brightly stains dead cells with compromised membranes. Cells were centrifuged at 1200 rpm, washed once with PBS and stained with the appropriate antibodies before being stained with Annexin V-FITC (Pharmingen). Cells were resuspended in 1ml of 1x Annexin binding buffer (Pharmingen) with 5μl of Annexin V-FITC and incubated in the dark at room temperature for 20 minutes. 5μl of Topro-3 iodide dye (Molecular Probes) was then added to each sample (final concentration of 5nM) before analysis on the flow cytometer. The following monoclonal antibodies and
second layer reagents were used: PE anti-CD4, PE anti-CD2, PE anti-Thy1.2, PE anti-CD44, biotinylated anti-CD8, biotinylated anti-mouse γδ T cell receptor (GL31), biotinylated anti-CD44 and anti-CD25 (Pharmingen), Tricolor conjugated anti-CD8, Tricolor conjugated anti-CD4 and streptavidin-Tricoliour (Caltag).

5.2.6B. Sub-G1 peak analysis by Propidium Iodide Staining

Duplicate cultures were performed and at each time point cells were also analysed using an assay to detect nuclear changes such as DNA fragmentation. Cells were centrifuged at 1200 rpm and washed once in PBS before being fixed in cold 70% ethanol. Ethanol was added dropwise to the cell pellet while gently vortexing to ensure all cells are fixed and to minimise clumping. Fixation was carried out for at least 30 minutes at 4°C. Cells were centrifuged at 2000 rpm and washed twice in phosphate-citrate buffer. Ribonuclease was added to final concentration of 0.5μg/ml and the cells were stained with propidium iodide (PI) (final concentration of 10μg/ml) for 20 minutes in the dark at room temperature. Samples were analysed on a flow cytometer and sub-G1 analysis was carried out.

Phosphate-citrate buffer 192 parts 0.2M Na₂HPO₄, 8 parts 0.1M citric acid

5.2.7. Intracellular stainings for the detection of the transgenic protein and also Bcl-2 and BclxL in the plck- Flag -RUNT transgenic mice

The population of interest was sorted and washed twice in PBS before being fixed in 1% paraformaldehyde for 20 minutes at room temperature. The cells were washed twice in PBS before being resuspended in 0.3% saponin buffer (SB),(0.3% saponin (SIGMA, St. Louis, MO), 10mM Hepes, 2% FCS, in PBS) and incubated for 10 minutes at room temperature. The cells were washed once in 0.3% SB before staining in 0.1% SB for 30 minutes at room temperature with the specific antibody or with the same concentration of control immune serum of the same IgG type as the specific antibody. The cells were washed twice in 0.1% SB before incubation for a further 30 minutes at room temperature with the appropriate FITC-conjugated immunoglobulin. Cells were then washed and stained with monoclonal antibodies to CD4 and CD8 if required and analysed as described previously. The following antibodies were used: hamster
anti-mouse Bcl-2 (Pharmingen), anti-BCLxL mouse monoclonal (IgG2b isotype, Transduction laboratories), polyclonal hamster IgG isotype standard (Pharmingen), mouse IgG2b isotype standard (Pharmingen), FITC labelled anti-hamster Ig and FITC-labelled anti-mouse Ig (Pharmingen). The expression of the Flag-RUNT protein in transgenic thymocytes was carried as described above using the following antibodies: an anti-Flag mouse monoclonal (IgG1, Santa Cruz; sc1656), mouse IgG1 isotype standard (Pharmingen), FITC-labelled anti-mouse Ig (Pharmingen).

5.3 Results

5.3.1. The expression of CBFα mRNA during early thymocyte development

To confirm previous work demonstrating that CBFα1 and CBFα2 are expressed throughout T cell development (Satake et al., 1995) and to precisely define the expression of endogenous CBFα mRNA during thymocyte development, Northern blot analysis was carried out. The probe used was specific for the RUNT domain of the CBFα subunit, allowing the expression of all three α subunits to be assessed. CBFα transcripts were present in RNA extracted from thymus and testes and were undetectable in lung and heart (Figure 5.1A). Cells from the four double negative (DN) thymocyte subsets, double positive (DP) and CD4 single positive (SP) cells were sorted and total RNA was extracted. CBFα transcripts were detected in each thymocyte subset (Figure 5.1B). When normalised to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, there was a slight upregulation of CBFα transcripts going from the first to the second DN subsets and a decrease in both the third and fourth DN subsets. The level of CBFα transcripts reached its maximum level in DP cells and was decreased in comparison in SP thymocytes. These results demonstrate that CBFα mRNA is transcribed at the earliest identifiable stage of T cell development and is present throughout thymocyte differentiation.
Figure 5.1: Northern blot analysis of the steady state level of CBFα mRNA in DN thymocyte subsets

Figure 5.1A: Total RNA was prepared from 10^6 thymocytes, lung, heart and testes to test the specificity of the CBFα probe. The CBFα probe was specific for the RUNT domain of murine CBFα1, so allowing for detection of all three CBFα proteins. The RNA samples were separated on a formaldehyde-agarose gel and the blots were hybridised with the indicated probes.

![Image of Northern Blot with Cbfα and GAPDH probes]

Figure 5.1B: Total RNA was prepared from 3 x 10^5 cells from each double negative (DN) thymocyte subset. Samples were treated as above. The intensity of the Cbfα band was quantitated using a phosphorimager and the values normalised against the GAPDH loading control. Abbreviations used: 1, CD44^+CD25^- DN1 cells; 2, CD44^+CD25^+ DN2 cells; 3, CD44^-CD25^+ DN3 cells; 4, CD44^-CD25^- DN4 cells; DP, double positive cells; SP, single positive cells.

![Image of Relative Quantitation Table]

<table>
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<th>Sample</th>
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<th>GAPDH (1.4Kb)</th>
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<tr>
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<td></td>
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<tr>
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5.3.2. Generation of CBF dominant negative transgenic mice

The dominant negative constructs used in these experiments are the DNA binding Runt domain of CBFα, that is common to all of the CBFα proteins, expressed under the control of the p56Lck proximal promoter. The transgenic protein, consisting of the DNA binding domain alone without the downstream transactivation domain, should compete with endogenous CBFα/β complexes for DNA binding sites and so reduce the activity of endogenous CBF activity. To increase the dominant interfering potential of the transgene, the RUNT domain was fused to the repressor domain of the Drosophila Engrailed transcription factor. This alanine rich region is a strong active repressor of transcription (Jaynes and O'Farrell 1991; Han and Manley 1993). Previous studies have shown that Engrailed-DNA binding domain chimeric molecules are able to repress the transcription of target genes more effectively than simple competitive inhibitors consisting of the DNA binding domain alone (Badiani et al., 1994). This RUNT-Engrailed sequence was then cloned into the p56Lck proximal promoter expression cassette (Chaffin et al., 1990). For identification purposes the protein is tagged with a myc epitope. A second construct was also generated to act as a control. The sequence for the RUNT domain in this construct contains a point mutation that has been shown to abolish its binding to DNA (Lee et al., 1997). These constructs are referred to as pLck-WT-RUNT-Engrailed and pLck-MUT-RUNT-Engrailed, respectively (see Figure 5.2 A and B).

To verify that the RUNT-Engrailed sequences can generate proteins, they were first cloned into pBluescript, which includes T7 promoter sequences. The resultant pBS-WT-RUNT-Engrailed and pBS-MUT-RUNT-Engrailed constructs were then transcribed and translated in vitro using the TnT T7 quick coupled reticulocyte lysate system (Promega). As shown in Figure 5.3, these constructs did not produce the expected polypeptide of approximately 51kDa. This was not due to technical difficulties since protein was clearly detected from the control DNA and from a mix of RUNT-Engrailed and control DNA. The RUNT-Engrailed DNA therefore did not inhibit the reaction, indicating an inherent problem with the design of construct. There are a number of possible reasons for
The pLck-RUNT-Engrailed constructs comprise the p56Lck proximal promoter (-3200 to +97 with respect to the transcription start site) fused to the wildtype RUNT sequence from the murine CBFα1 gene (A) or the mutant RUNT sequence (B) (Lee et al, 1997) plus the engrailed repressor domain and a myc-9E10 tag for identification purposes. A polyadenylation signal (pA+) and sequences for the human growth hormone (hGH) gene were added to the 3'end to enhance splicing and expression of the transgene (Chaffin et al, 1990).
Figure 5.3: *In vitro* translation of RUNT-Engrailed constructs

The pBS-WT-RUNT-Engrailed and pBS-MUT-RUNT-Engrailed constructs were subjected to *in vitro* translation using the TnT T7 quick coupled reticulocyte lysate system (Promega) to determine whether these constructs can generate transgenic protein of approximately 51kDa. 0.5µg of T7-luciferase DNA was used as a positive control (61kDa); in addition 0.25µg of pBS-WT-RUNT-Engrailed DNA was added to 0.25µg of this control DNA to ensure the DNA used did not inhibit the reaction.
this, although none of them have been substantiated. Transgenic mice generated previously using the engrailed repressor domain have fused the engrailed sequences directly downstream of the DNA-binding sequences and have placed the myc tag at the carboxyl terminal (Badiani et al., 1994; Taylor et al., 1996). It is possible that placing the myc tag in the centre of the chimeric molecule resulted in incorrect folding of the transgenic protein. In the future, if the Engrailed repressor was to be fused to the RUNT domain, or other DNA-binding domains to generate similar constructs, the myc tag will be placed at the carboxyl terminus of the protein. The construct was redesigned as described below.

5.3.3. Redesigning the CBF dominant negative construct

Following problems with the first construct the second was designed to be as simple as possible. Considering the work of Wang and colleagues it was decided that expression of the RUNT domain alone, without the Engrailed sequence, should interfere sufficiently with activity of endogenous CBFα (Wang et al., 1996c). The second construct was also designed taking into account recent work that identified a myc-related nuclear localisation signal (NLS) in CBFα1 (Thirunavukkarasu et al., 1998). This stretch of 9 amino acids (PRRHRQKLD) was found at the 3' end of the RUNT sequence and extends into downstream sequences. The second construct includes the full sequence of the RUNT domain and the NLS. A control construct including the mutated non-binding RUNT sequence was also generated (see Figures 5.4 A and B). For identification purposes the protein is tagged with a Flag-Tag (Kodak). These constructs will be referred to hereafter as pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT.

To verify that the Flag-RUNT sequences can generate proteins, they were cloned into pBluescript. The resultant pBS-WT-Flag-RUNT and pBS-Flag-MUT-RUNT constructs were then transcribed and translated \textit{in vitro} using the TnT T7 quick coupled reticulocyte lysate system (Promega). As shown in Figure 5.5, these constructs did produce the expected polypeptide of approximately 16.5kDa. The dominant negative activity of these constructs was then assessed before they were used to generate CBF dominant negative transgenic mice.
The pLck-Flag-RUNT constructs comprise the \( p56^{Lck} \) proximal promoter (-3200 to +97 with respect to the transcription start site) fused to the wildtype RUNT sequence from the murine CBF\( \alpha \)1 gene (A) or the mutant RUNT sequence (B) (Lee et al., 1997) plus a Flag-tag (F) for identification purposes. A polyadenylation signal (pA+) and sequences for the human growth hormone (hGH) gene were added to the 3' end to enhance splicing and expression of the transgene (Chaffin et al., 1990). The transgene was excised with Not1 prior to injection. Transgenic mice were identified by hybridisation of EcoR1 digested genomic Southern blots with a transgene-specific previously characterised hGH probe.
Figure 5.5: *In vitro* translation of Flag-RUNT constructs

The pBS-Flag-WT-RUNT and pBS-Flag-MUT-RUNT constructs were subjected to *in vitro* translation to verify that these constructs can generate transgenic protein of approximately 16.5kDa. 0.5μg of T7-luciferase DNA was used as a positive control (61kDa); in addition 0.25μg of pBSFlag-WT-RUNT DNA was added to 0.25μg of this control DNA to ensure the DNA used did not inhibit the reaction.
5.3.4. Assessment of the dominant negative activity of the Flag-RUNT proteins in vitro via transient transfection assays

Previous work has established the essential role of CBF in activation of transcription of the granulocyte macrophage-colony stimulating factor receptor (GM-CSF receptor) gene (Takahashi et al., 1995; Cockerill et al., 1996). These studies included transient transfection assays using GM-CSF receptor promoter/luciferase reporter gene plasmids (Cockerill et al., 1996). A GM-CSF receptor construct with a mutated CBF binding site (GM627ΔCBF) resulted in a three times reduced level of luciferase activity compared to the wild-type construct (GM627) with an intact CBF site. The GM627 and GM627ΔCBF constructs (gift from Dr P.Cockerill), here called wild-type GM-CSF and mutated GM-CSF, were used to assess the dominant negative activity of the Flag-RUNT constructs.

Before transient transfections were carried out the Flag-RUNT sequences were cloned into pCDNA3, which provides a higher level of expression of downstream sequences in mammalian cell lines than the p56Lck proximal promoter expression cassette. As shown in Figure 5.6, the mutated GM-CSF construct resulted in reduced luciferase activity, to an average of 40% of the level of the wild-type GM-CSF construct, so recapitulating published results. The luciferase activity of the wild-type GM-CSF construct was not affected by co-transfection with 4μg of a non-RUNT-expressing pCDNA3 control vector. GM-CSF luciferase activation was inhibited by increasing amounts of the Flag-WT-RUNT construct, with a level of less than 60% of the wild-type luciferase activity achieved when 4μg were used. Increasing amounts of the Flag-MUT-RUNT construct also inhibited GM-CSF luciferase activation, suggesting that this protein is also acting in a dominant negative fashion. As confirmed by sequencing this construct contains a RUNT sequence with a point mutation that has been reported to abolish its binding to DNA (Lee et al., 1997). One possible mechanism for this activity is that, although this mutated RUNT domain is reported as being unable to bind DNA, the protein should still be able to heterodimerize with the β subunit. Heterodimerization of the mutated RUNT
Calcium phosphate-mediated transient transfections were carried out using a total of 10µg of DNA. 2µg of either a wild-type (WT) or a mutated (Mut) GMCSF promoter/luciferase gene plasmid (which lacks the CBF binding site) plus the indicated quantities of either the pCDNA3-Flag-WT-RUNT or pCDNA3-Flag-MUT-RUNT construct were used (µg). In addition, 2µg of EGFP DNA was used as a control for transfection efficiency. The mean and standard deviation are presented from 5 sets of transfections.
protein to CBFB could result in interference with wild-type CBF\(\alpha\) function through competition for the CBFB subunit. Therefore, although the transgenic constructs did not consistently reduce the luciferase activity as much as mutating the CBF site, these results indicate that the Flag-WT-RUNT and the Flag-MUT-RUNT transgenic proteins can both interfere with the function of endogenous CBF\(\alpha/\beta\) complexes within these cells. Since subtle differences in the mode of action of these two dominant negatives may result in differential phenotypes \textit{in vivo} it was decided that the pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT mice should both be generated, so the activity of these transgenic proteins could be assessed.

5.3.5. Identification of transgenic founders by Southern blotting and Polymerase Chain Reaction (PCR)

Transgenic founders were identified by Southern blot hybridisation of genomic DNA extracted from tail snips and digested with EcoR1. EcoR1 cuts at the 5' and 3' end of the cloned transgene removing a 5.8kb fragment that can be detected by hybridisation with a transgene-specific human growth hormone (hGH) probe. In total seven founders were identified from one hundred and twenty mice screened. Figure 5.7 shows a southern blot identifying two pLck-Flag-WT-RUNT founders (LWR-1 and LWR-2) and three pLck-Ia-MUT-RUNT founders (LMR-1, LMR-2 and LMR-3). The relative copy number of (RCN) each of the lines was estimated by stripping the blots and re-hybridising with a probe specific for CBFA1, an endogenous gene present at a single locus. Using this probe, founder LWR-1 had a relative copy number of 12 and founders LWR-2, LMR-1, LMR-2 and LMR-3 had relative copy numbers of 1, 3, 6, and 2, respectively. Of the founders identified three pLck-Flag-WT-RUNT and three pLck-FLAG-MUT-RUNT founders were bred with C57BL/6-J mice, generating lines LWR-1, LWR-2 and LWR-3, and LMR-1, LMR-2 and LMR-3. The founder for line LWR-3 was sterile so this line was abandoned. Germline transmission was successful from the five other lines. Following initial identification of founders and establishment of founder lines, transgenic mice were identified using PCR. Primers specific for sequences within the p56\(^{Lck}\) proximal promoter
upstream of the cloning site and within the 3` end of the RUNT sequence were used, resulting in a 550 base pair product in transgenic mice (see Figure 5.8).

5.3.6. Northern blot analysis of the expression of the pLck-Flag-RUNT transgene.

Having successfully generated transgenic lines it is important to ensure that the pLck-Flag-RUNT constructs are being expressed within the transgenic thymocytes. Single cell suspensions of total thymocytes from the pLck-Flag-WT-RUNT (lines LWR-1 and LWR-2) and pLck-Flag-MUT-RUNT (line LMR-1) transgenic mice and from littermate controls were isolated and total RNA was extracted. Northern blot analysis was carried out using the RUNT/CBFα probe (previously used in Figure 5.1) to detect of transgenic and endogenous RUNT-containing CBFα transcripts within these thymocytes. The pLck-Flag-RUNT constructs were expressed, resulting in a transcript of approximately 1.3Kb (Figure 5.9). Upon normalisation to the level of glyceraldehyde 3-phosphate dehydrogenase mRNA the level of expression of the transgene is similar in all three lines. The level of transgenic mRNA is comparable to the level of endogenous CBFα mRNA.

5.3.7. Detection of the pLck-Flag-RUNT transgenic protein via intracellular staining and immunoblot analysis

The expression of transgenic protein was investigated via intracellular staining of thymocytes from wild-type, pLck-Flag-WT-RUNT and pLck-Flag MUT-RUNT mice, with an anti-Flag monoclonal antibody. The Flag-RUNT transgenic protein was detected in four of the five transgenics analysed, as shown by the shift in fluorescence in comparison to the isotype-matched serum control (figure 5.10). The second pLck-Flag-WT-RUNT (line LWR-2) did not express the Flag-tagged-RUNT protein. Wild-type thymocytes from C57BL/6 mice were used as a control and as expected showed no shift in fluorescence with the anti-Flag antibody.

In addition to simple detection of the transgenic protein it is also important to see where it is located in the cell. The transgenic construct was designed to be targeted to the nucleus to compete with endogenous CBFα protein. To determine whether this had been achieved, cytoplasmic and
Transgenic pLck-Flag-RUNT founders were detected by hybridisation of EcoR1 digested genomic Southern blots with the transgene specific human growth hormone (hGH) probe. Five pLck-Flag-RUNT founders were detected, each with transgene bands at 5.8Kb. The blots were then stripped and re-hybridised with a probe specific for CBFα1, an endogenous gene present at a single locus, to allow relative copy number (RCN) to be estimated. These founders were backcrossed with C57BL/6-J mice to establish transgenic lines LWR-1, LWR-2 (pLck-Flag-WT-RUNT lines) and LMR-1, LMR-2 and LMR-3 (pLck-Flag-MUT-RUNT lines).

Figure 5.8: PCR to genotype pLck-Flag-RUNT transgenic mice

Following initial identification of founders and establishment of founder lines, transgenic mice were identified using PCR. A "+" indicates a transgenic and a "-" indicates a non-transgenic littermate. A control reaction with distilled water (DW) instead of DNA was also performed. The primers and conditions used are described in section 5.2.2.
Figure 5.9: Northern blot analysis of the level of expression of the Flag-RUNT transgenic transcripts

Total RNA was prepared from $10^6$ thymocytes from pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT transgenic and from littermate control mice. The RNA samples were separated on a formaldehyde-agarose gel and the blots were hybridised with the $CBF\alpha$ probe previously used in Figure 5.1, to allow for detection of transgenic and endogenous $CBF\alpha$ RUNT-containing transcripts within these thymocytes. The intensity of the RUNT band was quantitated using a phosphorimager and the values normalised against the $GAPDH$ loading control. A "+" indicates a transgenic and "-" indicates a non-transgenic litter-mate control.

<table>
<thead>
<tr>
<th></th>
<th>LWR-1</th>
<th>LWR-2</th>
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<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>+</td>
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</table>

Endogenous $CBF\alpha$ (2.1Kb)

Transgenic $CBF/RUNT$ (1.3Kb)

$GAPDH$ (1.4Kb)

Relative quantitation for RUNT expression allowing for $GAPDH$ control:

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Figure 5.10: Detection of Flag-RUNT transgenic protein expression via intracellular staining and flow cytometric analysis

Total thymocytes from transgenic and littermate control mice (4-6 weeks of age) were stained intracellularly with an anti-Flag monoclonal antibody (Santa Cruz) (green lines) or with the same dilution of an isotype-matched control serum (purple lines).
nuclear protein extracts were prepared from pLck-Flag-RUNT transgenic mice and non-transgenic littermates and analysed via immunoblotting. The two lines chosen for this, and subsequent analysis, are lines LWR-1 and LMR-2. Thymocytes from these lines expressed high levels of the transgenic protein (refer to figure 5.10). A protein of approximately 16.5kDa was detected with the anti-Flag antibody in the nuclear lysates from the LWR-1 and LMR-2 transgenic thymocytes that was not seen in the cells from non-transgenic littermate controls (figure 5.11). A reduced amount of this protein was also detected within the cytoplasmic extracts of the transgenic thymocytes. Following incubation with the Flag antibody the blot was then stripped and immunoblotted with a control antibody. In this case an STAT5 monoclonal antibody was chosen since on activation this protein accumulates in the nucleus (Darnell et al., 1994). A similar level of STAT5 protein was detected within the nuclear extract of transgenic and non-transgenic mice alike but very little was detected within the cytoplasmic extracts. This confirmed that the samples contained a similar amount of protein and that the cytoplasmic and nuclear fractions were not cross contaminated. These results show that a considerable amount of the transgenic Flag-RUNT protein is being successfully targeted to the nucleus, where it should act in a dominant negative fashion to disrupt the normal function of CBF.

5.3.8. Fluorescence in situ hybridisation (FISH) analysis of the pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT transgenic lines

The integration site of a transgene can have important effects on its expression (see previous discussion in section 4.4.2). The location of the pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT transgenes in founder lines LWR-1 and LMR-2 respectively were identified by chromosomal FISH analysis. Figure 5.12 shows that each transgene has a single integration site which was on a different chromosome in each of the lines. Chromosomal paints reveal that the insertion site of the pLck-Flag-WT-RUNT transgene was located to chromosome 14 region C-D and the pLck-Flag-MUT-RUNT transgene was located to chromosome 7 region D.
Figure 5.11: Immunoblot analysis of pLck-Flag-RUNT protein expression in cytoplasmic and nuclear protein extracts

Cytoplasmic and nuclear protein extracts were prepared from $10^6$ thymocytes from pLck-Flag-RUNT transgenic mice (lines LWR-1 and LMR-2). Lysates were electrophoresed, transferred to a membrane and immunoblotted with an anti-Flag monoclonal antibody. The blot was then stripped and immunoblotted with a control antibody (an anti-STAT 5 monoclonal antibody) to ensure the extracts were not cross contaminated. A "+" indicates a transgenic and a "-" indicates a non-transgenic control mouse.
**Figure 5.12: Fluorescence in situ hybridisation (FISH) analysis of pLck-Flag-RUNT transgenic lines**

Localisation of Lck-GFP transgenic integration sites by chromosomal FISH to metaphase chromosomes. Transgene insertion was visualized using avidin-Texas Red after hybridisation with a biotinylated probe specific to *human growth hormone* sequences within the transgene. A diagrammatic representation of the location of the transgene (indicated by a green star) on an idiogram stained with Giemsa is shown for each transgenic line.

**WT Line LWR-1**

**MUT Line LMR-2**

**Chromosome 14 region C-D**

**Chromosome 7 region D**
5.3.9. Flow cytometric analysis of thymocytes, splenocytes and lymph node cells from pLck-Flag-RUNT transgenic and littermate control mice

To assess thymocyte development within the pLck-Flag-RUNT transgenic mice thymocytes, LN cells and splenocytes were isolated, stained with anti-CD4 and anti-CD8 monoclonal antibodies and analysed by flow cytometry. Thymocyte development was not arrested in the pLck-Flag-RUNT transgenic mice (Figure 5.13). The proportions of DN, DP and CD4 and CD8 SP thymocytes were similar in thymi from pLck-Flag-WT-RUNT, pLck-Flag-MUT-RUNT and wild-type mice. In the periphery the CD4/CD8 ratios of the pLck-Flag-RUNT transgenic mice were also comparable to those of wild-type mice. These results are representative of five independent experiments. This is confirmed in Figure 5.14 where the means and standard deviations from five mice are presented. Statistical analysis of these data using the Students t-test revealed that there are no significant differences with respect to thymiccellularity or CD4/CD8 ratios in the thymus or periphery between transgenic and wild-type mice.

Although thymocyte development is not arrested in the pLck-Flag-RUNT transgenic mice and thymic cellularity is normal in these mice, differences in the CD4/CD8 profiles were consistently seen between transgenic and non-transgenic littermates (see Figure 5.15). This was the case for the pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT mice. In every case when mice were analysed prior to determination of their transgenic status it was still possible to identify the transgenic from the non-transgenic littermates. The exact nature of these differences varied but in general the DP population was affected. The DP cells expressed a greater variation in the level of cell surface CD4 and CD8, resulting in a population with a more diffuse appearance on the FACS plots. During the experiments, it became clear that the severity of the phenotype depended on the treatment of the thymocytes. If the cells were treated harshly, for example, if they were not maintained on ice or were not analysed immediately, the differences between transgenic and non-transgenic mice thymocytes were exacerbated. These observations suggested that during the analysis the thymocytes from the transgenic mice might not be surviving as well.
Figure 5.13: Flow cytometric analysis of thymocytes, splenocytes and lymph node cells from pLck-RUNT transgenic and C57BL/6 control mice

Thymocytes, LN cells and splenocytes were isolated from 6 week old RUNT transgenic and littermate control mice, stained with anti-CD4 and anti-CD8 antibodies and analysed by flow cytometry. The results are representative of five experiments. Abbreviations used: T, Thymocytes; LN, Lymph node cells; SP, Splenocytes.
**Figure 5.14: Analysis of CD4/CD8 ratios in thymocytes, splenocytes and lymph node cells from pLck-Flag-RUNT transgenic and C57BL/6 control mice**

Thymocytes (A), splenocytes (B) and lymph node cells (C) were prepared from 6 week old pLck-Flag-WT-RUNT mice (line LWR-1), pLck-Flag-MUT-RUNT mice (line LMR-2) and non-transgenic control mice, stained with CD4 and CD8 antibodies and analysed by flow cytometry. Five mice were used for each genotype. Mean and standard deviation are presented.

<table>
<thead>
<tr>
<th>A</th>
<th>THYMIC CELLULARITY</th>
<th>CD4 SP (%)</th>
<th>CD8 SP (%)</th>
<th>CD4/CD8</th>
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<tr>
<td>Flag-WT RUNT</td>
<td>0.81 ± 0.317 x 10^8</td>
<td>9.96 ± 2.88</td>
<td>4.58 ± 1.38</td>
<td>2.29 ± 0.66</td>
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<td>Flag-MUT RUNT</td>
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<td>7.96 ± 3.34</td>
<td>3.01 ± 2.98</td>
<td>3.74 ± 1.28</td>
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<table>
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<tr>
<th>B</th>
<th>CD4 SP (%)</th>
<th>CD8 SP (%)</th>
<th>CD4/CD8</th>
</tr>
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<tr>
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<td>7.81 ± 0.43</td>
<td>3.21 ± 0.45</td>
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<tr>
<td>Flag-MUT RUNT</td>
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<td>6.37 ± 0.69</td>
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<tr>
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<table>
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<th>CD8 SP (%)</th>
<th>CD4/CD8</th>
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<td>2.88 ± 0.88</td>
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Figure 5.15: Further flow cytometric analysis of thymocyte development in pLck-Flag-RUNT transgenic mice

Thymocytes were isolated from litters of pLck-Flag-WT-RUNT mice, line LWR-1, (A) and pLck-Flag-MUT-RUNT mice, line LMR-2 (B), stained with anti-CD4 and anti-CD8 antibodies and analysed by flow cytometry. The boxes indicate cells with aberrant CD4/CD8 profiles. The results are representative of three independent experiments.
as those from wild-type mice. To see if there was any basis to these initial indications apoptosis assays will be carried out (section 5.3.12).

5.3.10. Flow cytometric analysis of the level of expression of αβTCR-CD3 complexes or γδTCRs in thymocytes from pLck-Flag-RUNT transgenic and littermate control mice

*In vitro* studies have revealed the presence and essential nature of CBF binding sites within the enhancers of the TCR chain genes. Mutation of these sites leads to a severe reduction in enhancer activity, revealing a crucial role for CBF in the regulation of TCR gene expression (Ogawa et al., 1993b; Giese et al., 1995). To investigate the effect of the dominant negative CBF transgene on the expression of the TCR genes thymocytes from pLck-Flag-RUNT-transgenic mice and non-transgenic littermate controls were stained with anti-αβTCR, anti-CD3ε or anti-γδTCR antibodies and analysed by flow cytometry. No differences in the percentage of cells expressing αβTCRs, CD3ε or γδTCRs were observed in the pLck-Flag-WT-RUNT or pLck-Flag-MUT-RUNT transgenics in comparison to non-transgenic littermate controls (figure 5.16). The level of expression of these cell surface proteins was also similar in transgenic and non-transgenic thymocytes. These results indicate that interference with CBF activity by the expression of the RUNT-transgene, in contrast to *in vitro* results, has no effect on the expression of the TCR complexes.

5.3.11. Flow cytometric analysis of foetal and neonatal thymocyte development

To investigate the possible role of CBFα in foetal thymocyte development embryos from pLck-Flag-RUNT transgenic mice and wild-type mice were again analysed for CD4 and CD8 expression. The results obtained for the pLck-Flag-WT-RUNT mice are presented here but analysis of the pLck-Flag-MUT-RUNT mice produced very similar results. Thymocytes were analysed from embryonic day (E) 15, 17, 19 and 21 (neonates), and from one-week-old mice. As shown in Figure 5.17, foetal and neonatal thymocyte development is not arrested in the pLck-Flag-RUNT transgenic mice. However, as seen in the thymus from adult transgenic mice, the DP population was more diffuse in the transgenic mice in
Figure 5.16: TCR expression during thymocyte development in the pLck-Flag-WT-RUNT transgenic mice and non-transgenic littermate controls

Thymocytes were prepared from 6 week old pLck-Flag-WT-Runt mice (line LWR-1) and control C57BL/6 mice, stained with anti-CD4, anti-CD8 and anti-αβTCR/anti-CD3/anti-γδTCR antibodies and analysed by flow cytometry. The results are representative of four experiments. Note that analysis of the Flag-MUT-RUNT mice (line LMR-2) revealed similar result. Abbreviations used: DN, double negative cells; DP, double positive cells; SP single positive CD4 or CD8 cells.

WT control  pLck-Flag-RUNT transgenic mice

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<th>DP</th>
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<th>CD8 SP</th>
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<tr>
<td>control</td>
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</table>

Number of cells (arbitrary scale)

αβ TCR

CD3

γδ TCR

Total thymocytes (following DN enrichment)
Figure 5.17: Foetal and neonatal thymocyte development in the pLck-Flag-WT-RUNT transgenic mice

Thymocytes were prepared from Flag-WT-RUNT transgenic mice (line LWR-1) and littermate control embryos and mice of the indicated ages, stained with anti-CD4 and anti-CD8 antibodies and analysed by flow cytometry. The red boxes indicate cells with aberrant CD4/CD8 profiles. The results are representative of four experiments. Analysis of the Flag-MUT-RUNT transgenic mice (line LMR-2) also revealed similar results.
comparison to non-transgenic littermates, due to a greater variation in the level of cell surface CD4 and CD8 expression in these cells. Thus, the transgenic approach used here indicates that CBFax does not have a major role in foetal and neonatal thymocyte development.

5.3.12. Reduced in vitro survival of thymocytes from the pLck-Flag-RUNT transgenics in comparison to wild-type controls

Flow cytometric analysis of thymocyte development in the pLck-Flag-RUNT mice indicated suggested that during analysis the transgenic thymocytes might not be surviving as well as those from wild-type mice. Following on from these initial indications apoptosis assays were carried out. Thymocytes from four to six week old transgenic mice and littermate controls were cultured in vitro with and without serum for up to 72 hours. Every 24 hours the percentage of apoptotic and dead cells was measured. Annexin-V was used to identify cells that were externalising their phospholipid phosphatidylserine (PS) residues as they underwent apoptosis. Dead cells were identified as those taking up Topro-3 iodide, a dye that is impermeable to the intact membranes of living cells. All of the following in vitro survival assays were performed with thymocytes from pLck-Flag-WT-RUNT (line LWR-1) and pLck-Flag-MUT-RUNT (line LMR-2) mice, producing similar results. The experiments using thymocytes from LWR-1 mice are presented here. Thymocytes from the pLck-Flag-RUNT transgenics showed a reduced survival rate compared to cells from wild-type mice when cultured with serum (figure 5.18A). After 24 hours 75% of non-transgenic thymocytes were still alive, with approximately 12% being apoptotic and 12% dead. In comparison at the same timepoint only 42% of the transgenic thymocytes were alive, with 27% being apoptotic and 30% dead. This pattern continued throughout the numerous time courses carried out, with the level of cell survival of pLck-Flag-RUNT thymocytes being nearly half that of wild-type cells. Thymocytes from the transgenic mice were also much more sensitive to serum deprivation than wild-type cells (see Figure 15.18B). After just 24 hours of starvation 70% of transgenic thymocytes had died in comparison to only 30% of wild-type cells. After 72 hours without serum the majority of both transgenic and wild-type cells were dead.
Figure 5.18 Apoptosis analysis of thymocytes from pLck-Flag-RUNT transgenic and littermate control mice by staining with Annexin V and Topro-3-iodide

Total thymocytes from pLck-Flag-RUNT mice (line LWR-1) and non-transgenic littermates were cultured in vitro with serum (A) or without serum (B) for the indicated times (hours), harvested, stained with Annexin-V and Topro-3 iodide and analysed by flow cytometry. The percentage of cells falling in each quadrant is indicated. These results are representative of four experiments. Abbreviations used: A, alive; D, dead; AP, apoptotic.
These results were confirmed by a second method for identifying apoptotic cells. When cells die or undergo apoptosis often they suffer DNA damage and loss that can be detected by staining the cells with propidium iodide. In this assay cells that have lost DNA fall into the sub-G1 peak and can be quantified. One limitation of this technique is that cells that have died without losing DNA from their nuclei will fall into the G1 peak and so not be detected. Sub-G1 analysis of thymocytes grown with serum for up to 72 hours revealed a higher level of cells with DNA loss in the pLck-Flag-RUNT transgenics in comparison to the wild-type cells. After 72 hours of culture with serum 35% of transgenic thymocytes had suffered DNA loss, as opposed to less than 6% of wild-type cells (Figure 15.19A). This analysis again revealed the increased sensitivity of pLck-Flag-RUNT transgenic thymocytes to serum deprivation in comparison to wild-type cells (Figure 15.19B). In addition to the increased number of cells falling into the sub-G1 peak in the transgenic mice, the cell cycle profiles also differ from those of wild-type mice at the G2 stage. The discrete peak of dividing cells seen in wild-type mice is significantly altered in the transgenic mice. This indicates that thymocytes are not progressing through the cell cycle normally in the transgenic mice due to an increased level of apoptosis. Thymocytes expressing the Flag-RUNT protein therefore have reduced viability in vitro, suggesting a role for CBFα in the regulation of cell survival and apoptosis.

5.3.13. Further analysis of the survival defect seen in pLck-Flag-RUNT transgenic thymocytes

To further understand the nature of the survival defect seen in the pLck-Flag-RUNT transgenic mice, thymocytes from these mice and from littermate controls, were cultured in vitro following a number of treatments. Cells were either untreated or were treated with TGF-β (final concentration of 1ng/ml, Human Platelets, Calbiochem), or Dexamethasone (final concentration of 0.1μM, Sigma), or were cultured in media without serum. The pLck-Flag-RUNT transgenic thymocytes showed reduced survival in comparison to wild-type cells when cultured with serum alone (Figure 5.20A). These differences were found to be significant (p=0.001). For thymocytes incubated without serum transgenic cells died more rapidly than wild-type cells (Figure 5.20B).
Figure 5.19 Sub-G1 analysis of thymocytes from pLck-Flag-RUNT transgenic and littermate control mice by staining with propidium iodide

Total thymocytes from pLck-Flag-WT-RUNT mice (line LWR-1) and non-transgenic littermates were cultured in vitro with serum (A) or without serum (B) for the indicated times (hours). The cells were then fixed with 70% ethanol and stained with propidium iodide. The percentage of cells falling in the sub-G1 peak is indicated. These results are representative of four independent experiments.
Figure 5.20 Further apoptosis analysis of thymocytes from pLck-Flag-RUNT transgenic and littermate control mice by staining with Annexin V and Topro-3 iodide

Total thymocytes from individual pLck-Flag-WT-RUNT mice (TG1, TG2 and TG3) and non-transgenic littermates (WT1, WT2 and WT3) were cultured in vitro with serum (A), without serum (B), following treatment with Dexamethasone (C) or with TGF-β (D) for the indicated times (hours), harvested, stained with Annexin-V and Topro-3 iodide and analysed by flow cytometry. These results are representative of three experiments.
These differences were also statistically significant ($p=0.009$). Extensive cell death was observed for both wild-type and pLck-Flag-RUNT transgenic thymocytes after treatment with Dexamethasone (Figure 5.20C). Due to the high sensitivity of both populations of thymocytes to this treatment it was not possible to detect any difference in the kinetics of cell death due to the presence of the pLck-Flag-RUNT transgene ($p=0.9$). Treatment with TGF-$\beta$ resulted in reduced survival of both wild-type and pLck-Flag-RUNT transgenic thymocytes in comparison to untreated cells ($p<0.002$) (Figure 5.20D). The overall difference in cell death kinetics between the transgenic and wild-type thymocytes was the same when the cells were treated with TGF-$\beta$ as when they were grown in serum alone. Therefore, the increase in cell death with TGF-$\beta$ treatment was merely additive to the differences already seen when the cells were grown in serum alone.

These results were confirmed by Sub-G1 analysis (Figure 5.21). The transgenic thymocytes were more susceptible to apoptosis when cultured with sera ($p=0.001$), or without sera ($p<0.001$) in comparison to wild-type thymocytes (Figure 5.21A and B). No significant differences in the rate of cell death between transgenic and control thymocytes could be detected on treatment with Dexamethasone (Figure 5.21C) ($p=0.28$). TGF-$\beta$ treatment resulted in minor increase in cell death of both transgenic and wild-type thymocytes, although by Sub-G1 analysis this was not significant (Figure 5.21D) ($p<0.15$). Again this increase in death of thymocytes was merely additive to the increase in cell death seen when these cells were grown in sera alone. Taken together, these results confirm that the presence of the pLck-Flag-RUNT transgene results in reduced survival of thymocytes in vitro. This survival defect is accentuated in the absence of serum. Therefore, a putative role for CBF in the regulation of thymocyte survival is implicated.
Figure 5.21 Further Sub-G1 analysis of thymocytes from pLck-Flag-RUNT transgenic and littermate control mice by staining with Propidium Iodide

Total thymocytes from pLck-Flag-WT-RUNT mice (TG1, TG2 and TG3) and non-transgenic littermates (WT1, WT2 and WT3) were cultured in vitro with serum (A), without serum (B), following treatment with Dexamethasone (C) or with TGF-β (D) for the indicated times (hours). The cells were then fixed with 70% ethanol and stained with propidium iodide. These results are representative of three experiments.
5.3.14. Analysis of the survival of different thymocyte sub-populations from the pLck-Flag-RUNT transgenic mice

Thus far, the survival of total thymocytes from wild-type and transgenic mice has been analysed. It is of interest to analyse cell survival within the different thymocyte subpopulations. As previously discussed, there are two main checkpoints in T cell development regulated by the expression of the pre-TCR and later the expression of a functional αβTCR. Cells that successfully rearrange and express a pre-TCR composed of the TCRβ chain, the pTα and CD3 components progress from the DN3 to DN4 stage. Cells that fail to express a functional pre-TCR do not develop into DN4 thymocytes but die by apoptosis apparently due to a lack of signalling from the pre-TCR (Penit et al., 1995). DN4 cells that survive the pre-TCR checkpoint proliferate and differentiate into DP cells. Following rearrangement of the TCRα chain genes and expression of an αβTCR these DP cells then undergo positive selection, negative selection or death by neglect based on the affinity of the αβTCR-CD3 complexes with MHC molecules. The majority of DP thymocytes die within the thymus by death by neglect or due to being negatively selected or failing positive selection.

To investigate whether the presence of the pLck-Flag-RUNT-transgene affects survival of all thymocyte subsets, regardless of their developmental stage, cells were again cultured in vitro without serum for 24 hours and were analysed for CD4/CD8 status prior to staining with Annexin-V and TORPRO-3-iodide (Figure 5.22A). When the cells are subdivided into the DN, DP and SP subsets there appears to be no differences with respect to survival between wild-type and transgenic cells in the DN compartment, with over 90% of cells from each mouse surviving regardless of the transgenic status. To confirm this result the DN3 and DN4 subpopulations were examined (Figure 5.22B). Thymi from four wild-type or four pLck-Flag-RUNT transgenic mice were pooled and depleted of cells expressing CD4 and/or CD8. The DN3 and DN4 populations were obtained via cell sorting and were cultured in vitro with serum for up to 72 hours. The results are representative of three independent experiments. No differences in DN3 or DN4 cell survival were detected between the wild-type thymocytes and the transgenic thymocytes in these experiments.
Figure 5.22 Further thymocyte survival analysis for pLck-Flag-RUNT transgenic and littermate control mice

A: Total thymocytes from 4 week old pLck-Flag-WT-RUNT mice (line LWR-1) and non-transgenic littermate control mice were cultured \textit{in vitro} without serum for 24 hours, stained with anti-CD4 and anti-CD8 antibodies, Annexin-V-FITC and Topro-3 iodide and analysed by flow cytometry to assess the cell survival in different thymocyte subsets.

B: The appropriate thymocyte populations were obtained via cell sorting and were cultured \textit{in vitro} with serum for the indicated times (hours). The cells were then harvested and stained with Annexin-V-FITC and Topro-3 iodide and analysed by flow cytometry. The results are representative of three independent experiments.
Analysis of the DP population revealed that the reduced survival of pLck-Flag-RUNT transgenic thymocytes is due to a reduced survival of DP cells. On average over 60% of DP thymocytes from wild-type mice survive after culture for 24 hours without serum but survival of DP cells is reduced to less than 40% in the pLck-Flag-RUNT transgenic mice (Figure 5.22A). This result was also confirmed after DP cells were obtained by cell sorting and cultured for up to 72 hours in the presence of serum (Figure 5.22B). After 48 hours approximately 50% of wild-type DP thymocytes survived but over 95% of the pLck-Flag-RUNT transgenic DP thymocytes have died. The presence of the transgene had little affect on the survival of SP cells since the survival of CD4 and CD8 cells from wild-type and pLck-Flag-RUNT transgenic mice were similar (Figure 5.22A). The expression of the dominant negative CBF transgene therefore results in increased levels of apoptosis in DP cells. This indicates a role for CBF transcription factors in the regulation of DP cell survival, either through the transactivation of genes promoting cell survival or through repression of the transcription of pro-apoptotic genes.

5.3.15. Analysis of the expression of Bcl-2 and BclxL anti-apoptotic proteins in pLck-Flag-RUNT transgenic and littermate control mice

The CBF transcription factors are important regulators of expression of a number of T cell specific genes, as well as other genes involved in haematopoiesis and bone development. The genes regulated by CBF that are involved in thymocyte survival are as yet unknown. There are multiple pathways that lead to apoptosis of thymocytes, involving a number of different families of genes. One of the first families of genes identified to regulate cell survival/death was the Bcl-2 family. The level of expression of two anti-apoptotic family members, Bcl-2 and BclxL, in the pLck-Flag-RUNT-transgenic mice has been investigated. Single cell suspensions of total thymocytes from pLck-Flag-RUNT transgenic mice and their non-transgenic littermates were stained intracellularly for Bcl-2, BclxL, or with a control sera and analysed by flow cytometry. The wild-type and pLck-Flag-RUNT transgenic thymocytes showed similar levels and patterns of expression of both Bcl-2 and BclxL protein (figure 5.23). In agreement with published data Bcl-2 protein is expressed at
Figure 5.23: Flow cytometric analysis of thymocytes from pLck-Flag-RUNT transgenic and littermate control mice stained intracellularly for Bcl-2 and Bcl-X<sub>L</sub>.

Total thymocytes from pLck-Flag-WT-RUNT transgenic mice (line LWR-1) and non-transgenic littermates were stained with anti-CD4 and anti-CD8 monoclonal antibodies before staining intracellularly with monoclonal antibodies against Bcl-2, Bcl-X<sub>L</sub> or with control immunoglobulins of the appropriate isotype. Abbreviations used: DN, double negative cells; DP, double positive cells; SP single positive CD4 or CD8 cells.
high levels in the DN cells, is downregulated in DP thymocytes and upregulated in CD4 and CD8 SP cells (Veis et al., 1993; Moore et al., 1994). Bclx\textsubscript{L} protein shows a distinct pattern of expression, being expressed at a maximal level in DP thymocytes from wild-type and transgenic mice, again agreeing with published data (Grillot et al., 1995; Ma et al., 1995). The reduced survival of thymocytes expressing the CBF dominant negative transgene does not therefore seem to be due to an altered level of Bcl-2 or Bclx\textsubscript{L}.

5.4. Discussion

The CBF family of heterodimeric transcription factors play important roles in haematopoiesis and osteogenesis. This activity is mediated by the DNA binding RUNT domain of the CBF\textalpha partner that recognises a specific DNA sequence in numerous genes whose products are vital for these processes. The RUNT sequence also mediates interactions of the \alpha subunit with other proteins, including the non-DNA binding \beta subunit (Kagoshima et al., 1993; Meyers et al., 1993; Ogawa et al., 1993b; Kagoshima et al., 1996). Although the \beta subunit does not bind to DNA itself it is essential for the function of CBF\textalpha \textit{in vivo} (Wang et al., 1996c). The CBF\textalpha genes are expressed throughout thymocyte development, impicating a role for the CBF family in thymocyte differentiation (Satake et al., 1995). In addition, binding of the heterodimeric CBF\textalpha/\beta complex to gene enhancers has been shown to be required for the transcriptional activation of numerous T cell specific genes, including those that encode for chains of the TCR, for CD2 and CD3\textepsilon (Krimpenfort et al., 1988; Ho et al., 1989; Gottschalk and Leiden 1990; Lake et al., 1990; Hallberg et al., 1992; Redondo et al., 1992; Wang and Speck 1992; Hsiang et al., 1993; Ogawa et al., 1993b). CBF\textalpha2 and CBF\textbeta deficient mice die between embryonic day 11.5 and 12.5 due to haemorrhaging in the CNS and lack definitive haematopoiesis (Okuda et al., 1996; Sasaki et al., 1996; Wang et al., 1996b; Niki et al., 1997). In addition, CBF\textalpha2 deficient ES cells failed to contribute to haematopoiesis in chimeric animals (Okuda et al., 1996). Therefore, the role of CBF proteins in T cell development and function has not been elucidated through studies of these mice. Thymocyte development was
normal in the \textit{CBFα1} deficient mice, suggesting that other family members are involved or that the role of \textit{CBFα1} in thymopoiesis is redundant (Otto et al., 1997). To investigate the role of the heterodimeric \textit{CBFαβ} complex in thymocyte differentiation we have generated transgenic mice expressing a dominant negative form of \textit{CBF} within developing thymocytes. The results presented here have indicated a novel role for \textit{CBF} in thymocyte survival. The potential mechanisms for this activity, and putative downstream genes targeted by \textit{CBF} in its role as a regulator of thymocyte apoptosis, are discussed.

5.4.1. Generation of \textit{CBF} dominant negative transgenic mice

5.4.1A. Design of the \textit{CBF/RUNT} transgenic constructs

As previously described, there are reasons to believe that \textit{CBFαβ} levels are limiting, therefore the expression of low levels of a competing protein should effectively reduce the level of functional \textit{CBF} within these cells and allow its role in thymocyte development to be analysed (Wang et al., 1996c). The dominant negative constructs used here were based on the expression of the DNA binding \textit{RUNT} domain of \textit{CBFα} under the control of the \textit{p56\textsuperscript{Lck}} proximal promoter (Figure 5.2). The \textit{CBF/RUNT} transgenic protein should compete with binding of endogenous \textit{CBFαβ} complexes for DNA binding sites and act in a dominant negative way due to lacking the transactivation domain of \textit{CBFα} (Bae et al., 1994; Ducy and Karsenty 1995; Aronson et al., 1997). Since the \textit{Runt} domain is common to all of the \textit{CBFα} proteins expression of this construct should target all three \textit{CBFα} proteins and so overcome functional redundancy between these family members. The described transgenic \textit{RUNT} proteins also lack the recently identified negative regulatory region for heterodimerization (NRH or QA region), that prevents heterodimerization with the \textit{β} subunit (Thirunavukkarasu et al., 1998), and the negative regulatory region for DNA binding (NRBD) (Kanno et al., 1998) (see figure 5.24). The transgenic proteins should therefore be able to heterodimerize with the \textit{β} subunit more readily than endogenous \textit{CBFα} and also bind to DNA more readily. In addition, a second construct with a mutated \textit{RUNT} sequence has been generated (Lee et al., 1997). The transgenic protein produced by this construct should be unable to bind to
DNA, so transgenic mice generated with this construct were designed to be control mice (see section 5.4.1D).

![Figure 5.24: A diagrammatic representation of the functional domains of CBFα2 protein](image)

Above is a diagrammatic representation of the structure of full-length CBFα2. Numbers denote the positions of amino acids. The DNA-binding Runt domain is shown (Kagoshima et al., 1993; Meyers et al., 1993; Ogawa et al., 1993b; Kagoshima et al., 1996), as is the region responsible for nuclear matrix association (Zeng et al., 1997). Other abbreviations used are as follows: AD; transcription activation domain (Kanno et al., 1998), ID; transcription inhibition domain (Kanno et al., 1998), NRDBn and NRDBc; negative regulatory region for DNA-binding, located at N-terminal and C-terminal sides respectively (Kanno et al., 1998; Kim et al., 1999b), NRHn and NRHc; negative regulatory region for heterodimerization, located at N-terminal and C-terminal ends respectively (Lu et al., 1995; Thirunavukkarasu et al., 1998), NLS; nuclear localisation signal (Thirunavukkarasu et al., 1998), Smad 1, 2, 3, 5; the region responsible for interaction with the Smad proteins (Hanai et al., 1999), VWPRY; the sequence required for association with the TLEs (Aronson et al., 1997) and Hes-1 indicates the carboxyl region required for interaction with Hes-1 (McLaren et al., 2000).

5.4.1B. Choice of transgenic promoter for the CBF-dominant negative transgenic construct

As described in the results section, CBFα mRNA is detectable in the earliest T cell progenitors in the thymus and present throughout thymocyte development (Figure 5.1). Therefore, the dominant negative transgene needs to be expressed early in thymocyte development so as to compete with the endogenous CBFα/β complex. For example, it is known that CBF is required for the efficient activity of the TCRβ enhancer (Ogawa et al., 1993b). It is therefore important that the dominant negative CBF/RUNT protein is available to compete with endogenous CBF at the time when this enhancer is normally active. It is within the CD44^+CD25^- (DN2) population that rearrangement of the TCRβ loci is initiated resulting in the detection of Dβ-Jβ transcripts within these
cells. Full length V(D)J TCR\(\beta\)transcripts are detectable by the CD44^CD25^ (DN3) stage of development (Godfrey et al., 1993). It was therefore decided that the RUNT transgenes be expressed under the control of the p56\(^{Lck}\) proximal promoter. From the generation of pLck-GFP transgenic mice we had gained a number of insights into the use of this promoter (Chapter 4). The pattern of GFP expression varied between different pLck-GFP lines and did not correlate with the relative copy number (RCN) of the transgene (Figures 4.7 and 4.8). This implied that the p56\(^{Lck}\) proximal promoter lacks locus control region (LCR) activity (Figure 4.10). Transgenes expressed downstream of promoters with incomplete or absent LCR activity can be subject to position effects such as PEV (for review see Karpen 1994). This can result in random silencing of expression of transgenes integrated near/within regions of heterochromatin in a proportion of cells. Transgenes are also affected by regulatory elements at the site of integration, which can enhance or repress transgene expression. Despite these integration-dependent effects, GFP expression was consistently observed from the DN3 population and was even seen as early as the DN1 population depending on the founder line analysed (Figure 4.8). Only one pLck-GFP line (line LG-1), where the transgene had integrated near to the centromere (a region of constitutive heterochromatin), lacked expression in DN1 and DN2 thymocytes. Fluorescence in situ hybridisation (FISH) analysis of the LWR-1 and LMR-2 transgenic lines revealed a single transgene integration site, away from the centromere and telomere, for both lines (Figure 5.12). This indicates that these transgenes may be integrated in a reasonably open area of chromosome and so should not be subject to PEV. The p56\(^{Lck}\) proximal promoter driven Flag-RUNT transgenic proteins should therefore be expressed early enough to act as effective dominant negative proteins.

5.4.1C. Expression and localisation of the pLck-Flag-RUNT transgenic protein

As already mentioned the timing of expression of a transgene can be an important factor in elucidating the role that a gene plays in a developmental process. In addition, since the Flag-RUNT proteins are designed to act as dominant negative proteins competing with endogenous CBF\(\alpha/\beta\) complexes for binding sites, the level of expression can also be a limitation. In this case the pLck-Flag-WT and the pLck-Flag-MUT-RUNT transgenes were both expressed
at similar levels to endogenous CBFα transcripts (Figure 5.9). Assuming translational efficiencies are the same for the transgenic and endogenous transcripts, there should be approximately equal amounts of the dominant negative transgenic protein and the endogenous protein available to bind to each CBF binding site. Recent work has revealed that RUNT-alone transgenic proteins bind DNA with a higher affinity and form more stable complexes than the full-length CBFα1 (Ducy et al., 1999). Thus, the transgenic protein should be able to successfully compete with the binding of the endogenous CBFα/β complex, if not completely ablate its activity.

In order to function as a dominant negative molecule, the transgenic protein has to be targeted to the nucleus, where it can compete with endogenous CBFα/β protein. Figure 5.11 demonstrates that the RUNT protein is detected in the nuclear lysates of the pLck-Flag-WT-RUNT and pLck-Flag-MUT-RUNT transgenic mice and not detected in the non-transgenic littermate controls. Therefore, the transgenic Flag-RUNT protein is being successfully targeted to the nucleus where it can block the normal function of the CBFα/β complex, thus acting in a dominant negative fashion. Transgenic protein was also detected in the cytoplasmic lysates, particularly from the pLck-Flag-MUT-RUNT mice. The pLck-Flag-RUNT constructs were designed to include sequences for the recently identified CBFα1 myc-related nuclear localisation signal (NLS) (Thirunavukkarasu et al., 1998). This stretch of 9 amino acids (PRRHRQKLD) is found at the 3' end of the RUNT sequence and extends into downstream sequences (see figure 5.24). Transient transfection assays using a wild-type protein construct and a construct with an in-frame deletion of this motif in the full-length coding sequence (OsfΔNLS) have been carried out (Thirunavukkarasu et al., 1998). Immunoblot analysis revealed the presence of the wild-type protein predominantly in the nucleus. In contrast, the OsfΔNLS protein was found only in the cytoplasm. The presence of transgenic protein in the cytoplasmic extracts from the pLck-Flag-MUT-RUNT transgenic thymocytes may represent protein that, although initially targeted to the nucleus, has, due to its small size, passively diffused back into the cytoplasm due to not being actively retained.
5.4.1D. Assessment of the dominant negative activity of the RUNT transgenic protein

Transient transfection assays have confirmed that the proteins generated by the pLck-Flag-RUNT constructs can act in a dominant negative fashion, resulting in decreased activity of the granulocyte macrophage colony stimulating factor (GM-CSF) receptor promoter (Figure 5.6). The Flag-WT-RUNT transgenic protein consistently resulted in the activity of this promoter being reduced to approximately half of its original level. Although the transgenic constructs did not consistently reduce the luciferase activity as much as mutating the CBF site, these results indicate that the Flag-WT-RUNT transgenic protein can interfere with the function of endogenous CBFα/β complexes within these cells. Surprisingly, the Flag-MUT-RUNT construct also resulted in a reduction of the activity of the GM-CSF receptor promoter, despite the fact that this protein contains a mutated, non-DNA-binding RUNT sequence (Figure 5.6) (Lee et al., 1997). It is possible that, although this mutated RUNT domain is reported as being unable to bind DNA, the protein can still heterodimerize with the β subunit. Heterodimerization of the α subunit with the β subunit is mediated by the RUNT sequence (Kagoshima et al., 1993; Meyers et al., 1993; Ogawa et al., 1993b; Kagoshima et al., 1996). The transgenic proteins lack the recently identified negative regulatory region for heterodimerization (NRH region) that prevents heterodimerization with the β subunit and so should be able to heterodimerize with the β subunit more readily than endogenous CBFα (Thirunavukkarasu et al., 1998). Work by Wang and colleagues has shown that the CBFβ subunit is essential for CBFα2 to function in vivo (Wang et al., 1996c). Therefore, successful heterodimerization of the mutated RUNT protein to CBFβ could still result in interference with wild-type CBFα function through competition for the CBFβ subunit. It is also possible that this mutated-protein maintains a limited ability to bind DNA, sufficient to act in a dominant negative manner.

The wild-type and the mutated RUNT transgenic proteins block the function of endogenous CBFα/β complexes to a similar degree. This suggests that the original hypothesis that the Flag-WT-RUNT protein would act by blocking DNA binding sites may not be a complete explanation. In addition to.
directly competing with endogenous CBFαβ for DNA binding sites the wild-type transgenic protein may also be acting by sequestering the β subunit from the endogenous α subunit. Furthermore, previous work has demonstrated that CBFα1 positively regulates its own promoter due to the presence of CBF binding sites within the CBFα1 gene promoter (Ducy et al., 1999). This suggests that the RUNT-transgenic protein may also bind to CBFα1 sites in the CBFα1 promoter and inhibit transcription of endogenous CBFα1. There is no evidence of this in the pLck-Flag-RUNT transgenic mice since the levels of endogenous RUNT-containing transcripts are approximately the same in transgenic thymocytes and wild-type thymocytes. However, differences in expression levels of CBFα1 may not be detected here since the RUNT probe recognises all three CBFα isoforms. Recent work has suggested that CBFα1 may not be the major thymic isoform since CBFα1 mRNA is expressed at a twenty-times higher level in bone than in thymus (Banerjee et al., 1997; Ducy et al., 1997). Thus, the mechanisms by which the Flag-RUNT transgenic proteins act to block the normal function of the CBFαβ protein are not fully understood. It was hypothesised that subtle differences in the mode of action of the two transgenic proteins might result in transgenic mice with differential phenotypes. However, as described in the results sections no phenotypic differences have been detected between these transgenic lines. In conclusion, the approach used here has successfully generated at least a partial dominant negative transgenic model with which to study the function of CBF in thymocyte development.

5.4.2. Explanations for the lack of a T cell development phenotype in the pLck-Flag-RUNT transgenic mice

As demonstrated in Figures 5.13 to 5.17, thymocyte development is essentially normal in the pLck-Flag-RUNT transgenic mice, both during foetal and adult life. Thymocyte cellularity is normal in these mice, as are the proportions of DN, DP and CD4 and CD8 SP thymocyte subsets. In the periphery the CD4/CD8 ratios of the pLck-Flag-RUNT transgenic mice were also comparable to those of wild-type mice. However, in foetal and adult transgenic mice the DP thymocyte population had a more diffuse appearance on the FACS plots than the DP thymocytes from non-transgenic littermates, due to
a greater variation in the level of cell surface CD4 and CD8 expression in these cells. The differences between transgenic and non-transgenic mice thymocytes were exacerbated when the cells were treated in a harsh manner, suggesting that during the analysis the thymocytes from the transgenic mice might not be surviving as well as those from wild-type mice. To further investigate these findings apoptosis assays were carried out (see section 5.4.3).

Thymocyte development was therefore not arrested in the pLck-Flag-RUNT transgenic mice. In addition, the expression of αβTCR-CD3 complexes or γδTCRs appears to be unaffected by the presence of the transgenic protein (Figure 5.16). This is somewhat surprising at first given the crucial role of CBF in the transcriptional activation of the TCRα, β, δ and γ enhancers in vitro. CBF binding sites are frequently adjacent to binding sites for other transcription factors, such as CREB, Ets-1, LEF-1 and AP-1 in enhancer sequences and CBF has been shown to co-operate with these factors to facilitate transcriptional activation. In vitro mutagenesis studies of the binding sites for these various transcription factors have revealed that they are required for effective TCR gene enhancer activity (see section 5.1.3 and Ogawa et al., 1993b; Wotton et al., 1994; Giese et al., 1995). It was originally hypothesised that interference with CBF activity may result in thymocyte development being arrested at the DN3 thymocyte stage due to cells failing to express a TCR. However, this study suggests that CBF is not required in vivo for the expression of the TCR chain genes, or at least that the reduction in activity of CBF via the expression of the dominant negative is not sufficient to affect their expression.

Gene targeting and transgenic studies investigating the transcription factors that CBF co-operates with suggest that the absence of a more major defect in thymocyte development is not so surprising. For example, transgenic mice expressing a dominant negative from of CREB have been generated (Barton et al., 1996). Thymocyte development is not perturbed in these mice, thymocyte number is normal and thymocytes expressed normal levels of CD3 and αβTCR. Thymocytes and T cells from these mice have other defects (see section 5.4.3.2). CREB deficient mice have also been generated which die at birth
(Rudolph et al., 1998). In contrast to the CREB dominant negative transgenics, thymic cellularity is severely reduced in the CREB-deficient embryos and thymocyte development is impaired, with a general increase in DN and a decrease in DP thymocytes. TCRα chain expression is not altered in CREB deficient mice and TCRβ chain expression is only mildly affected. The discrepancy between the normal thymocyte development in the CREB transgenic line and the severely affected development in the CREB-deficient mice may be due to the different genetic approaches used. Homologous recombination results in inactivation of CREB in early precursors entering the thymus whereas expression of a dominant negative occurs later in development. In addition, the role of Ets-1 in T cell development has been investigated by the generation of Ets-1-deficient RAG-2 chimeric mice (Bories et al., 1995; Muthusamy et al., 1995). The T cells in these mice expressed normal levels of CD3 and αβTCR but were present in reduced numbers. The above in vivo studies suggest that, in contrast to in vitro findings, that a certain level of redundancy may exist between the transcription factors involved in activation of expression of the TCR chain genes (Anderson et al., 1989; Gottschalk and Leiden 1990). Functional redundancy has been demonstrated for two HMG (high-mobility group) proteins, T cell factor 1 (Tcf-1) and lymphoid enhancer-binding factor 1 (Lef-1) that also regulate expression of the TCRα gene enhancer. Targeted gene disruption of either the Tcf-1 or the Lef-1 gene did not affect TCRα gene expression and resulted in an incomplete defect or no defect in thymocyte differentiation (van Genderen et al., 1994; Verbeek et al., 1995). However, thymocyte development in double mutant mice deficient for Tcf-1 and Lef-1 is completely blocked at the immature single positive (ISP) stage (Okamura et al., 1998). These ISP cells show a severely reduced level of TCRα gene expression. Therefore, due to compensation by other transcription factors and limitations of the targeting strategy an essential role for CBF in the expression of the TCR chain genes and in thymocyte development has not been elucidated.

5.4.3. The link between CBF and apoptosis

The pLck-Flag-RUNT transgenic mice are not arrested in thymocyte development but their thymocytes show reduced survival in vitro and an increased sensitivity to serum starvation (Figures 5.18 to 5.22). This phenotype is
not widespread in vivo probably due to removal of apoptotic cells by macrophages. This indicates a role for CBF transcription factors in the regulation of thymocyte survival, either through the transactivation of genes promoting cell survival or through repression of transcription of pro-apoptotic genes. This finding is in agreement with previous work demonstrating that overexpression of CBFα2 results in protection from apoptosis in T cell hybridoma cell clones (Fujii et al., 1998). Analysis of the survival of the different sub-populations of thymocytes revealed that the presence of the pLck-Flag-RUNT-transgene did not affect the survival of DN3 or DN4 sub-populations (see Figure 5.22). Rather, the reduced survival of pLck-Flag-RUNT-transgenic thymocytes was shown to reflect an increased level of apoptosis within the DP thymocyte compartment. This fits with the initial observations of variations within the DP subset, with transgenic thymocytes expressing somewhat varying levels of CD4 and CD8 (Figure 5.17). The CBF transcription factors are therefore implicated in the regulation of the survival of DP thymocytes through the transactivation or repression of downstream target genes.

A novel role for the CBF family of transcription factors in the regulation of thymocyte survival has been identified. As already mentioned, there are multiple pathways that are involved in the survival of thymocytes. We have investigated the expression of two members of the Bcl-2 family, Bcl-2 and BclxL, which are important promoters of cell survival (see Figure 5.23). The levels of these two proteins were not altered in the pLck-Flag-RUNT transgenics in comparison to wild-type cells. The reduced survival of thymocytes expressing the CBF dominant negative transgene is therefore not due to an altered level of Bcl-2 or BclxL.

5.4.3.1. Potential apoptotic pathways involving CBF

Extensive research has been carried out to identify genes involved in the numerous pathways controlling cell survival within the thymus. Of these pathways those involving CBF are as yet unidentified. A number of possibilities will now be discussed.
5.4.3.1A. Interleukin 7 signalling

A crucial role for interleukin 7 (IL-7) in T cell development, through the regulation of cell proliferation and survival, has been identified. Mice deficient for IL-7 exhibit a profound decrease in overall thymic cellularity and an increased percentage of apoptotic cells within the thymus (von Freeden-Jeffry et al., 1995; von Freeden-Jeffry et al., 1997). Evaluation of the Bcl-2 status of DN thymocytes from IL-7 deficient mice revealed that the DN2, DN3 and DN4 cells from these mice lacked expression of this anti-apoptotic gene, which is normally highly expressed at these developmental stages. Enforced expression of the Bcl-2 gene in thymocytes from interleukin 7 receptor α chain (IL-7Ra) deficient mice, through introduction of the Eμ-Bcl-2 transgene, resulted in rescue of thymopoiesis in these mice (Akashi et al., 1997). These data suggest that IL-7 functions in thymocyte survival through regulating the expression of Bcl-2. This has been confirmed by studies culturing DN thymocytes with and without IL-7. In the absence of IL-7 DN1, DN2 and DN3 cells die rapidly by apoptosis, whereas DN4 cells grow independently of this cytokine (Kim et al., 1998). Analysis of the expression of Bcl-2 and its pro-apoptotic family member Bax, revealed that IL-7 treatment resulted in increased expression of Bcl-2 and decreased expression of Bax in these cells. A critical role for IL-7 for thymocyte survival and development prior to the rearrangement of the TCR, mediated by control of expression of Bcl-2 family members, has therefore been identified. The functions of this cytokine later in thymocyte development are currently less well understood. The reduced survival of thymocytes expressing the CBF dominant negative transgene is not due altered levels of expression of Bcl-2 or Bclx_l. Also the survival defect seen in the pLck-Flag-RUNT transgenic mice affects DP thymocytes but not those at earlier stages of development, so it is unlikely that CBF is linked to IL-7 signalling in its role in thymocyte survival.

5.4.3.1B. Pre-TCR signalling

Genetic targeting studies and the generation of transgenic mice have revealed the essential nature of the pre-TCR in thymocyte development and survival. Cells that successfully rearrange and express a pre-TCR composed of the TCRβ chain, the pTα and CD3 components progress from the DN3 to DN4 stage. This transition between DN3 to DN4 cells is the first major checkpoint in
development at which thymocytes are susceptible to apoptosis. Thymocytes that survive the pre-TCR checkpoint proliferate and differentiate into DP cells. Mice that are deficient for recombination activator genes (RAG 1 and 2) and SCID mice are blocked in thymocyte development at the DN3 stage, due to failing to rearrange their TCRβ chain genes (Mombaerts et al., 1992b; Shinkai et al., 1992) (Blunt et al., 1995; Kirchgessner et al., 1995). Similarly, thymocyte differentiation is arrested at this stage in CD3ε deficient mice (Malissen et al., 1995; Wang et al., 1998b). Thymocyte development in pTα deficient mice is also partially arrested at this point, although a low percentage of DP cells develop (Fehling et al., 1995). Thymocytes from the above mutant mice and normal mice that fail to express a functional pre-TCR are thought to die by apoptosis due to a lack of signalling from the pre-TCR (Penit et al., 1995). Recent work has revealed that the combined effects of pre-TCR signalling and death receptor signalling are important for determining whether a DN3 thymocyte survives, proliferates and differentiates or whether it dies by apoptosis. Death receptors, including members of the tumour necrosis factor receptor (TNF-R) family such as Fas, possess cytoplasmic death domains. These domains mediate interactions with death domain-containing cytoplasmic adapter proteins such as FADD. Introduction of a transgenic dominant negative mutant of FADD into RAG-1 deficient mice resulted in the generation of DN4 and DP thymocytes (Newton et al., 2000). The authors of this work postulated that in the absence of pre-TCR signalling, such as in cells with non-productive TCRβ chain rearrangements, that DN3 cells die by apoptosis following death receptor signalling. Whereas, in DN3 cells that have successfully expressed a functional TCRβ pre-TCR signalling blocks the apoptotic signal and the cell survives. The identity of the death receptors involved in the propagation of these death signals via FADD are unknown. However, T cell development remains blocked at the DN3 stage in RAG-1 deficient mice that are homozygous for the Fas loss of function lpr mutation, indicating that Fas is not involved (Newton et al., 2000). It is possible that CBF is involved in signalling downstream of the pre-TCR. However, the DN4 thymocytes from the pLck-Flag-RUNT transgenic mice are not increasingly susceptible to apoptosis so implicating that pre-TCR signalling is unaffected in these mice.
5.4.3.1C. Positive and negative selection

DN4 cells that survive the pre-TCR checkpoint proliferate and differentiate into DP cells. Following rearrangement of the TCRα chain genes and expression of an αβTCR these DP cells then undergo positive selection, negative selection or death by neglect based on the affinity of the αβTCR-CD3 complexes with MHC molecules. The majority of DP thymocytes have negligible affinity for the various MHC-associated peptides found within the thymus and die within a few days by death by neglect. The remaining cells undergo the processes of positive or negative selection due to specificity for thymic MHC/peptide complexes. Negative selection eliminates from the thymus DP cells with αβTCRs that react with high affinity to self-peptide/MHC within the thymus. Negative selection phenotypes can be difficult to detect due to the small fraction of thymocytes that actually undergo negative selection. Thymocytes with αβTCRs that recognise self MHC with low affinity are positively selected, rescued from death by neglect and survive and differentiate into TCR high CD4 or CD8 SP T cells (for review see Saito and Watanabe 1998). It is possible that the reduced survival of thymocytes expressing the CBF dominant negative transgene is due to altered levels of positive or negative selection or death by neglect. The links between CBF and aspects of thymic selection that have been described will now be discussed.

Glucocorticoid receptor signalling. A potential link between CBF and the glucocorticoid receptor (GR) signalling pathway exists. DP thymocytes are known to be highly sensitive to glucocorticoid treatment, even the physiological concentrations achieved during a stress response can be sufficient to result in apoptosis of these cells (Cohen and Duke 1984; Gruber et al., 1994). The vast majority of thymocytes die within the thymus and endogenous glucocorticoids are, at least in part, thought to be responsible for eliminating cells that die by death by neglect. The production of glucocorticoids, such as pregnenolone and deoxycorticosterone, by thymic epithelial cells has been documented (Vacchio et al., 1994). These small lipophilic compounds diffuse across the plasma membrane and contact the intracellular GRs of adjacent thymocytes. On ligand binding these GRs translocate to the nuclei and regulate the transcription of
glucocorticoid responsive genes. In addition to a potential role in death by neglect, glucocorticoids have been implicated in positive selection of thymocytes, although this has been a matter of some debate. These complex actions of glucocorticoids result in part from mutual antagonism of glucocorticoid and TCR mediated signalling pathways (Ashwell et al., 2000). Glucocorticoids are thought to be involved in the positive selection of thymocytes that recognise self-MHC with low affinity by antagonising TCR signals that would otherwise result in cell death. In this context, inhibition of glucocorticoid receptor (GR) signalling results in an increase in TCR mediated antigen-specific deletion of thymocytes (Vacchio et al., 1994). In addition, inhibition of glucocorticoid synthesis in thymic organ cultures resulted in apoptosis of thymocytes that would normally be positively selected (Vacchio and Ashwell 1997). Negative selection of thymocytes with high affinity to self-antigen/MHC occurs since these signals are too strong to be overcome by glucocorticoids.

A link between CBFα1 and glucocorticoid signalling exists since treatment of osteoblasts with hydrocortisone/Dexamethasone resulted in a reduction of CBFα1 mRNA expression and suppression of bone formation (Chang et al., 1998). To further investigate the relationship between CBF and GR signalling in thymocytes wild-type and pLck-Flag-RUNT transgenic thymocytes were treated with the pharmacological glucocorticoid Dexamethasone and the rate of cell survival was analysed. Both the wild-type and the pLck-Flag-RUNT transgenic thymocytes died rapidly on treatment with Dexamethasone. No changes in the kinetics of cell death were seen between the wild-type and the transgenic thymocytes (Figure 5.20C and 5.21C). A difference in cell survival between wild-type and transgenic thymocytes on treatment with Dexamethasone is very difficult to detect from these studies due to the high sensitivity of thymocytes to this treatment (Cohen and Duke 1984). Slight variations in the kinetics of cell death cannot be seen when so many cells are dying so rapidly. Although no conclusions as to the relationship between the GR signalling and CBF can be made from these data a link between these two is a potential explanation for the survival defect of the pLck-Flag-RUNT transgenic
thymocytes. The increased sensitivity of the pLck-Flag-RUNT transgenic DP thymocytes to apoptosis following culture and serum starvation indicates that these cells are not responding well in vitro to these stressful conditions. *In vivo* thymocytes expressing the CBF/RUNT transgene may also be increasingly susceptible to glucocorticoids produced during stress responses. If GR signalling results in reduced CBFα expression in thymocytes it can be envisaged that in the transgenic cells, which effectively already have reduced CBFα/β activity, the threshold for glucocorticoid induced death is lowered. Further work studying the effects of naturally occurring glucocorticoids, such as corticosterone, at a more physiological concentration is needed to test this hypothesis.

**Fas/Fas-L induced cell death.** A potential link between CBF and Fas/Fas-L induced cell death has also been described. The Fas receptor belongs to the Tumour Necrosis Factor (TNF) receptor family and Fas/Fas-L induced cell death has been shown to be important for deletion of activated mature T cells (for review see Ashkenazi and Dixit 1998). The role of Fas/Fas-L cell death within the thymus is unclear. Fas-L is predominantly expressed on activated T cells but is also detected within the thymus (Suda et al., 1995). Fas is expressed at a low level on DN thymocytes but is highly expressed on DP and SP thymocytes (Ogasawara et al., 1995). Fas-L binds to Fas and induces apoptosis of Fas bearing cells. DP thymocytes have been seen to selectively undergo apoptosis upon treatment with an anti-Fas antibody (Ogasawara et al., 1995). Negative selection was originally described as normal in the naturally occurring Fas receptor mutant *lpr* mice and Fas-L mutant *gld* mice (for review see Nagata and Suda 1995). However, recent reports indicate a role for Fas in negative selection when antigen is expressed in the thymus at a high level (Kishimoto et al., 1998; Kurasawa et al., 1999). The selective death of DP thymocytes in the pLck-Flag-RUNT transgenic mice could reflect increased negative selection due to enhanced sensitivity to Fas/Fas-L induced cell death. Studies utilizing T hybridoma cell clones that overexpress CBFα2 have demonstrated a link between CBF and Fas/Fas-L induced cell death (Fujii et al., 1998). Overexpression of CBFα2 resulted in a reduced level of Fas-L mRNA expression within these cells and a corresponding resistance to apoptosis. If
CBFα/β complexes are normally involved in the regulation of Fas-L expression in thymocytes then expression of the dominant negative CBF/RUNT transgene could result in overexpression of Fas-L and enhanced Fas/Fas-L induced cell death of transgenic thymocytes. Further work, investigating the expression of Fas and Fas-L within transgenic thymocytes, is needed to test this hypothesis.

In conclusion, the increased susceptibility of DP thymocytes from the pLck-Flag-RUNT transgenic mice to apoptosis indicates a role for CBF in the processes that control the fate of DP cells. The vast majority of DP thymocytes die within the thymus from death by neglect, failing positive selection or through being negatively selected. Therefore it is possible that CBF is linked with thymic selection processes in some way and that the reduced survival of thymocytes expressing the CBF dominant negative transgene is due to altered levels of positive or negative selection or death by neglect.

5.4.3.1D. A link between CBF and Transforming Growth Factor-β signalling pathways

Publications from a number of groups have indicated a link between CBF and the Transforming Growth Factor β (TGF-β) signalling pathway. The TGF-β family of cytokines are important regulators of biological processes through modulation of the expression of target genes. TGF-β signalling is mediated by two types of transmembrane serine/threonine kinase receptors from which signals are transduced to the nucleus by proteins of the Smad family (Kawabata and Miyazono 1999). TGF-β binds to the type II receptor on the cell surface which then heterodimerizes with the type I receptor. The constitutively activated type II receptor phosphorylates and activates the type I receptor that in turn phosphorylates and activates Smad proteins. Activated Smad proteins translocate to the nucleus where they act, in conjunction with other transcription factors (including CBF), to regulate the expression of target genes. These include genes encoding for cell cycle regulators, adhesion proteins and extracellular matrix (ECM) proteins. Through the modulation of expression of these various genes TGF-β regulates numerous biological processes including cell adhesion, to growth arrest, and apoptosis. The roles of TGF-β signalling in thymocyte differentiation, survival and proliferation are not well understood.
CBFα1 has been shown to bind to the TGF-β type I receptor promoter and to be important for regulation of its expression (Ji et al., 1998). In addition, CBFα1 has been shown to function synergistically with Smad3 to activate TGF-β signalling pathway (Hanai et al., 1999). Studies have also revealed that overexpression of Smad2 results in enhanced Smad4 expression and a reduction of CBFα1 mRNA levels in an osteoblast-like cell line and in primary rat calvaria cells (Li et al., 1998). To investigate the relationship between CBF and TGF-β signalling pathways, wild-type and pLck-Flag-RUNT transgenic thymocytes were treated with TGF-β and the rate of cell survival was analysed. Treatment with TGF-β resulted in both wild-type and pLck-Flag-RUNT transgenic thymocytes dying at a slightly increased rate than cells treated with serum alone (Figure 5.20D). The pLck-Flag-RUNT transgenic thymocytes were equally sensitive to TGF-β induced accelerated death as wild-type thymocytes since the increase in cell death with TGF-β treatment was merely additive to the differences already seen when the cells were grown in sera alone. The roles of TGF-β signalling in thymocyte development are not well understood and it is not possible to draw any conclusions from these experiments. TGF-β has been implicated in the control of cell survival and of cell death and its pro and anti-inflammatory effects have been described (for review see Wahl, 2000). The increased susceptibility of thymocytes to cell death seen here when they are treated with TGF-β implies a possible role for TGF-β in apoptosis. However, the phenotype of TGF-β deficient mice implies that TGF-β acts in some way to protect thymocytes from apoptosis. These mice show twice the level of apoptosis in their DP and SP thymocytes compared to wild-type controls due to increased levels of Fas and Fas-L and increased susceptibility of changes in mitochondrial membranes (see Wahl et al., 2000). No conclusions as to the relationship between TGF-β signalling and CBF can be made due to the preliminary nature of the data presented here and the dearth of information regarding the function of TGF-β signalling in the thymus.
5.4.3.2. Other transgenic and knock-out models with similar apoptotic phenotypes

A recent paper, describing transgenic mice that express full-length \( CBF\alpha1 \) under the control of the CD2 promoter, has confirmed a role for \( CBF \) in thymocyte survival (Vaillant et al., 1999). The CD2-\( CBF\alpha1 \) transgenic mice were generated in order to study the oncogenic potential of a lymphoma-specific \( CBF\alpha1 \) isoform upon expression within the thymus. The \( CBF\alpha1 \) isoform expressed within the thymus of the CD2-\( CBF\alpha1 \) transgenic mice varies from the form expressed in normal T cells at its N-terminus. Therefore, the authors state that it is possible that this transgene will act in a dominant negative fashion. This study describes a low incidence of spontaneous lymphoma in the CD2-\( CBF\alpha1 \) transgenic mice with 6% of mice developing tumours between 107 – 315 days of age, whereas no littermate controls succumbed to neoplastic disease. Upon crossing these mice with transgenic lines carrying \( c-myc \) transgenes a dramatic increase in incidence and decrease in latency of tumour development was seen. Therefore, this indicates that full-length \( CBF\alpha1 \) can co-operate with the \( c-myc \) gene product in lymphomagenesis. Analysis of the thymic phenotype of CD2-\( CBF\alpha1 \) transgenic mice reveal both similarities and differences between these and the \( pLck-Flag-RUNT \) transgenic mice presented here (Vaillant et al., 1999). Neither transgene results in a change in overall thymic cellularity or in a block in thymocyte differentiation. The CD2-\( CBF\alpha1 \) transgene does, however, result in a skew towards CD8 SP cells in the thymus. This CD8 skew is not maintained in the peripheral T cell compartment. Thymocytes from both sets of transgenic mice show reduced survival upon culture in vitro. In contrast to thymocytes from the \( pLck-Flag-RUNT \) transgenic the CD2-\( CBF\alpha1 \) thymocytes showed a slightly increased sensitivity to TGF-\( \beta \) treatment in comparison to littermate controls. The CD2-\( CBF\alpha1 \) protein includes the region of \( CBF\alpha \) responsible for interaction with the TGF-\( \beta \) signal transducing Smad proteins which is lacking from the Flag-RUNT transgenic protein (see figure 5.24) (Hanai et al., 1999). This may explain the enhanced sensitivity of thymocytes from the CD2-\( CBF\alpha1 \) mice, but not the \( pLck-Flag-RUNT \) transgenic mice, to TGF-\( \beta \) induced cell death. The kinetics of cell death in response to Dexamethasone treatment is not altered by the presence of either
transgene. These results imply the full length CBFα-G1 transgenic protein may also be acting as a dominant negative molecule, resulting in reduced survival of thymocytes expressing this protein.

As previously described, CBF co-operates with transcription factors, such as Ets-1 and CREB, to regulate the expression of the TCR genes. Interestingly, studies that have disrupted the function of these transcription factors have also revealed roles for these proteins in thymocyte survival. For example, thymocytes and T cells from CREB dominant negative transgenic mice show a proliferative defect, characterized by markedly decreased IL-2 production (Barton et al., 1996). These cells are also more susceptible to apoptosis and G1 cell cycle arrest following a number of stimuli. Ets-1 deficient T cells also displayed a severe defect in proliferation and an enhanced susceptibility to apoptotic cell death (Muthusamy et al., 1995). The similarities between these phenotypes make it tempting to speculate that CBF may, through multiple protein-protein interactions, be involved in a complex with these other transcription factors that functions to regulate the expression of genes involved in control of thymocyte survival.

5.5. Conclusions and future work

The work presented here investigated the role of the CBF transcription factors in thymocyte development using a transgenic approach. Through the generation of dominant negative CBF transgenic mice a role for CBF in thymocyte survival has been elucidated. The potential mechanisms for this activity and putative downstream genes targeted by CBF however remain unidentified. Future work aims to further investigate these aspects of the function of CBF within the thymus.

It is clear from the discussion within this thesis how investigations into thymocyte development have benefited from gene targeting and transgenic studies. Despite the immense amount of information that has been gleaned from such transgenic studies there are a number of disadvantages to this technology, several of which have been alluded to here. The forced expression of genes
under the control of heterologous tissue-specific promoters can result in disruption of cellular processes in ways that are not fully understood. Differences in the level and timing of expression between the endogenous gene and the transgene, as well as phenomena such as Position Effect Variegation (PEV) can severely limit the use of information obtained by such studies (for review see Basson and Zamoyska 2000). If this project were to be repeated, more recently established technologies could be utilised. For example, the Cre/loxP recombination system could be used to inactivate the common CBFβ gene within thymocytes (for a review of advances in gene targeting methods, see Kuhn and Schwenk 1997). This would involve the generation of mice with the CBFβ gene flanked with loxP sites. These mice could then be crossed with transgenic mice expressing Cre under a T cell specific promoter, to result in inactivation of CBFβ within thymocytes.

5.5.1. The CBF transcription factors as regulators of TCR gene expression

Analysis of TCR expression of pLck-Flag-RUNT transgenic thymocytes revealed, in contrast to in vitro findings, that this transcription factor might not be essential for TCR chain gene expression. As discussed, this may be due to limitations in the reduction of endogenous CBFα/β activity achieved via the dominant negative transgene or may result from redundancy between CBF and other transcription factors also involved in the regulation of the TCR genes. Homozygous pLck-Flag-RUNT transgenic mice will be generated and analysed since these should express a higher level of the transgene. To investigate the level of redundancy between the transcription factors involved in the regulation of the expression of the TCR chain genes it would be of interest to breed the pLck-Flag-RUNT and the dominant negative CREB transgenic mice together (Barton et al., 1996). Due to interference with the function of both CBF and CREB, these double transgenic mice may be arrested in thymocyte development at the stage of TCR chain gene expression.
5.5.2. Identification of the genes targeted by CBF transcription factors in its role the regulation of thymocyte survival

The expression of the pLck-Flag-RUNT transgenic protein results in an increased susceptibility of DP thymocytes to apoptosis. Since CBF is a transcriptional regulator, the simplest model is that this apoptotic phenotype results from interference with the endogenous activity of CBF which would normally induce the expression of one or more gene products involved in the regulation of thymocyte survival. The expression of two members of the Bcl-2 family, Bcl-2 and BclxL which are important promoters of cell survival have been investigated. The levels of these two proteins were not altered in the pLck-Flag-RUNT transgenics in comparison to wild-type cells. Future work aims to identify the downstream genes targeted by CBF in its role as a regulator of thymocyte apoptosis. Representational Difference Analysis (RDA) will be carried out to identify genes that are differentially expressed in DP thymocytes from the pLck-Flag-RUNT transgenic mice in comparison to DP cells from wild-type mice. In conjunction with this work, RDA will be carried out on the immature SCB T cell line (derived from SCID mice). Previous work has demonstrated that overexpression of CBFα1 in non-osteoblastic cells induces the expression of the principle osteoblast-specific genes (Ducy et al., 1997). Thus, overexpression of full-length CBFα2 within SCB cells may allow for the identification of gene products whose expression is induced following CBF expression in these immature T cells.

5.5.3. The role CBF transcription factors in thymic selection events

To investigate the role of CBF in thymic selection events the pLck-Flag-RUNT transgenic mice are being bred with H-Y αβTCR transgenic mice. Thymocytes expressing the H-Y-specific transgenic αβTCRs are positively selected in female H-2^b mice and negatively selected in male H-2^b mice (von Boehmer 1990). This system will allow the effect of the pLck-Flag-RUNT transgene on thymic selection processes, which might be quite subtle due to the small number of cells undergoing particularly negative selection, to be analysed.

Previous work has indicated a link between CBF and GR signalling in osteoblasts. At this stage, due to time constraints, apoptosis assays have not
been successfully carried out here to investigate this relationship within thymocytes. However, a link between CBF and GR signalling is a potential explanation for the survival defect of the pLck-Flag-RUNT transgenic thymocytes. Despite extensive analysis, the mechanism(s) by which glucocorticoids cause apoptosis are still largely unknown. Glucocorticoid-induced genes that mediate cell death remain to be identified and, although gene products that can block glucocorticoid-induced cell death have been described, these pathways are not fully characterised. This therefore complicates future work that could be potentially carried out to investigate the effects of manipulation of GR signalling in transgenic versus wild-type thymocytes since downstream targets are not known. However, experiments will be performed to confirm the link between CBF and GR signalling within the thymus. Foetal thymic organ cultures (FTOCs) will be carried out, initially with wild-type thymi. The effect of treatment with glucocorticoids (for example, the water-soluble cholesterol analogue 22R-hydroxycholesterol, ICN) and with commercially available inhibitors of steroidogenic enzymes (for example, the P450c11 inhibitor Metrapone, ICN) on the expression levels of CBF will be assessed. Once the link between CBF and GR signalling is established, the relationship between CBF, these signalling pathways and apoptosis can be further investigated with similar experiments using thymocytes from pLck-Flag-RUNT transgenic and wild-type mice.

The expression of Fas and Fas-L within transgenic thymocytes will also be investigated to substantiate the involvement of Fas/Fas-L induced cell death in the thymic survival defect of the pLck-Flag-RUNT transgenic mice.

5.5.4. Analysis of the incidence of lymphoma in the pLck-Flag-RUNT transgenic mice

Interference with the normal function of CBF, by chimeric proteins generated due to chromosome alterations of the $CBF\alpha_2$ and $CBF\beta$ genes can contribute to the development of leukaemia. In addition, the expression of a lymphoma-specific isoform of CBF$\alpha_1$ (potentially acting in a dominant negative fashion) within the thymus results in an increased incidence of spontaneous lymphoma (Vaillant et al., 1999). In the CD2-CBF$\alpha$-G1 transgenic mice 6% of
mice develop T cell tumours between 107 – 315 days of age, whereas no littermate controls succumbed to neoplastic disease. The incidence of thymic lymphoma within the pLck-Flag-RUNT-transgenic mice, as well as non-transgenic littermates, is being monitored to investigate if the presence of the pLck-Flag-RUNT-transgene predisposes these mice to cancer.

5.5.5. Investigations into which CBFα isoform is involved in thymocyte survival

As mentioned, the RUNT sequence is common to all CBFα isoforms so the defect in thymocyte survival seen in the Flag-RUNT transgenic mice could be due to interference with the function of CBFα1, 2 or 3. It is therefore not clear which of these three isoforms are of importance in thymocyte survival. Cbfα1 and Cbfα2 have been described as being expressed throughout thymocyte development, whereas the expression pattern of CBFα3 is less well documented. The fact that CBFα1 mRNA has been identified in bone at a twenty-times higher level than in thymus (Banerjee et al., 1997; Ducy et al., 1997) and is essential for bone development (Komori et al., 1997; Otto et al., 1997) implies that this isoform may not be of importance in thymocytes. To investigate this, thymocytes from CBFα1 deficient embryos will be obtained and thymocyte survival assays performed as described above. If this isoform is not required for thymocyte survival these CBFα1 deficient thymocytes will show no difference in survival in comparison to wild-type and heterozygous litter-mate controls. If however this isoform is required for the control of apoptosis within thymocytes these CBFα1 deficient cells may have a more severe survival defect. The lack of definitive haematopoiesis in the CBFα2 and CBFβ deficient mice precludes similar studies with cells from these mice.
Chapter 6

General Discussion

The development of mature, antigen specific T cells from uncommitted precursor cells within the thymus is a tightly controlled, multistage process. The progression of immature thymocytes through this complex series of selection events can be followed phenotypically by the expression of certain cell surface markers, including CD4, CD8 and CD3. The advancement of thymocytes from one stage to the next requires the successful completion of specific developmental processes controlled by signals that activate lineage specific genes, allowing cellular proliferation, survival and differentiation. Gene targeting experiments, the generation of transgenic mice and studies of several human deficiencies have greatly contributed to our understanding of the stages of thymocyte development. Such studies have enabled intracellular signalling molecules involved in thymocyte development to be identified and their roles in the activation of expression of downstream genes, including transcription factors, to be further dissected. The work presented within this thesis focused on the regulation and function of two genes coding for molecules specifically involved in T cell development.

There are two main developmental checkpoints within thymocyte differentiation: the progression from the DN to DP stage and from the DP to SP stage. These checkpoints are regulated by the surface expression and downstream signalling of the pre-TCR and the mature αβTCR respectively. The signals propagated by these receptors are regulated by two families of protein tyrosine kinases (PTKs), the Src and the Syk families. The first gene investigated here was the p56Lck gene that encodes a lymphoid specific Src family PTK. p56Lck was first discovered due to being over-expressed in a Moloney murine leukaemia virus-transformed lymphoma cell line LSTRA. Gene targeting experiments and the generation of transgenic mice have demonstrated a vital role for this PTK in thymocyte development. These studies have also indicated the importance of tight control of its expression since inappropriate expression
of p56^Lck can lead to malignancies. Despite the vital role for p56^Lck in thymocyte development and the common usage of its proximal promoter to drive the expression of transgenes within thymocytes, little is known about the control of its expression in the thymus. To investigate the developmental timing of p56^Lck we determined the expression of transcripts and protein in each of the DN thymocyte subsets. In addition, we generated transgenic mice expressing green fluorescent protein (GFP) under the control of the p56^Lck proximal promoter. Our results showed that the expression of the p56^Lck gene is developmentally regulated at the post-transcriptional level at the precise developmental stage at which its upstream receptor complex, the pre-TCR, is expressed. The possible mechanisms of this control have been discussed. Analysis of several independent pLck-GFP transgenic mouse lines in this study revealed a number of insights into the use of the p56^Lck proximal promoter. This promoter was shown to be T cell specific, with a high level of activity in thymocytes and also a certain level of activity in peripheral T cells. Analysis of the pLck-GFP mice demonstrated that the expression of at least one transgene product under the control of the p56^Lck proximal promoter can vary between transgenic lines and does not always faithfully recapitulate the expression of endogenous p56^Lck mRNA or protein. These studies failed to find any evidence for locus control region (LCR) activity within the p56^Lck proximal promoter. This underscores the importance of the analysis of multiple transgenic founder lines when using transgenic mice to investigate the consequences of tissue specific expression of a gene. These results have implications for the use of the p56^Lck proximal promoter in the generation of transgenic mice and also for the interpretation of the phenotypes of previously generated transgenic animals that utilised the p56^Lck proximal promoter.

The second gene that was investigated was the gene coding for the heterodimeric transcription factor, Core Binding Factor (CBF). CBF is a heterodimer of an α, DNA binding subunit which is a product of one of three distinct genes and a common β subunit. Translocations targeting the CBFα2 and CBFβ genes are amongst the most frequent mutations in human leukaemia. The
α subunits contain a conserved 128 amino acid DNA binding domain, known as the runt domain because of its high homology to the Drosophila pair-rule gene runt. Via this runt domain CBF heterodimeric proteins bind the enhancer core sequence (TGYGGT), a sequence motif that is present in transcriptional enhancers of a variety of T cell specific genes, including the enhancers of the TCR αβ and δ-chain genes and the CD3e-chain. The presence of CBF binding sites within these T cell specific genes and their expression throughout thymocyte development supports the notion that the CBF family of transcription factors play a role in T cell development. CBFα1, CBFα2 and CBFβ deficient mice have been generated. The role of CBF proteins in T cell development and function has not been elucidated through studies of these mice. CBF dominant negative transgenic mice were generated in order to investigate the role of these transcription factors in thymocyte development. Initial analysis of the CBF dominant negative transgenic mice revealed thymi of normal size, the presence of normal numbers of DN, DP and SP thymocyte populations and normal levels of αβTCR–CD3 and γδTCR expression. In vitro apoptosis assays revealed that thymocytes from the transgenic mice showed reduced survival and increased sensitivity to serum-starvation in comparison to wild-type thymocytes. The results presented here have therefore indicated a novel role for CBF in thymocyte survival. The potential mechanisms for this activity and the as yet unidentified downstream genes targeted by CBF in its role as a regulator of thymocyte survival have been discussed.
References


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