School of Biomedical Sciences

The Role of Conserved Lymphokine Element 0 in Induction and Inhibition of Interleukin-5

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This thesis is presented for the Degree of Doctor of Philosophy of Curtin University of Technology

March 2004
DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

Signature:

Date:
ABSTRACT

The role of eosinophilia in allergic disorders indicates hIL-5 as a target of therapy. The conservation of hIL-5 proximal elements suggests they are important in controlling expression. Corticosteroids are important in the treatment of allergy, and are powerful inhibitors of IL-5 expression. Antisense oligonucleotides are new compounds that can specifically inhibit IL-5 production. This study aimed at understanding the role of conserved lymphokine element 0 (CLE0) in induction and inhibition of IL-5.

The conserved proximal CLE0/TATA elements driving a luciferase reporter gene gave higher expression than a 500bp promoter in PER117 T-cell line. Two and three copies of IL-5 CLE0 upstream of the silent IL-4 minimal promoter gave 150-200 fold increases in expression in forward orientation, but little activity in reverse orientation. Consequently, while CLE0 is a powerful activator, it is not a classical enhancer. Antisense technology has also shown the dependence of IL-5 gene transcription on the de novo synthesis of the transcription factor Fra2.

Inhibition of IL-5 reporter constructs by dexamethasone when induced by PMA/cAMP, but not PMA/CaI, provided a tool for understanding the mechanism. Deletion analysis identified CLE0 as the key element of dexamethasone inhibition. Non-inhibition of IL-5 reporter constructs by dexamethasone in a Jurkat cell line, however, showed a possible intermediary factor involved in the inhibition mechanism.
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCDF₁</td>
<td>B-cell differentiation factor ₁</td>
</tr>
<tr>
<td>BCGFII</td>
<td>B-cell growth factor II</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium²⁺</td>
</tr>
<tr>
<td>Cal</td>
<td>Calcium Ionophore</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic-adenosine-monophosphate</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>CLE0</td>
<td>Conserved Lymphokine Element 0</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony-stimulating factor</td>
</tr>
<tr>
<td>ECP</td>
<td>Eosinophil cationic protein</td>
</tr>
<tr>
<td>EDF</td>
<td>Eosinophil differentiation factor</td>
</tr>
<tr>
<td>GCS</td>
<td>Glucocorticosteroid</td>
</tr>
<tr>
<td>GILZ</td>
<td>Glucocorticoid-induced leucine zipper</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GRE</td>
<td>Glucocorticoid response element</td>
</tr>
<tr>
<td>HES</td>
<td>Hypereosinophilic syndrome</td>
</tr>
<tr>
<td>hIL-5</td>
<td>Human IL-5</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
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<td>Interleukin-5</td>
</tr>
<tr>
<td>IL-5R</td>
<td>Interleukin-5 receptor</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mIL-5</td>
<td>Murine IL-5</td>
</tr>
<tr>
<td>NF-AT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohemagglutinin</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PRE1</td>
<td>Palindromic regulatory element 1</td>
</tr>
<tr>
<td>PRE2</td>
<td>Palindromic regulatory element 2</td>
</tr>
<tr>
<td>rhIL-2</td>
<td>Recombinant human interleukin-2</td>
</tr>
<tr>
<td>RPH</td>
<td>Royal Perth Hospital</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TRF</td>
<td>T-cell replacing factor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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CHAPTER 1

INTRODUCTION
1.1 Interleukin-5 and Eosinophilia

Eosinophilia is a specific phenomenon that involves elevation in eosinophil numbers often in the absence of changes in any other cell compartment. Eosinophils are formed in the bone marrow from precursors in response to cytokine activation, and are released into the circulation following an appropriate stimulus. During circulation, eosinophils accumulate rapidly in tissue, where they synthesize and release mediators that can cause oedema, bronchoconstriction and chemotaxis. They also secrete enzymes and proteins that can damage tissue (Greenfeder et al., 2001). In asthmatics, the presence of eosinophils in the lung is associated with tissue damage and remodelling (Desreumaux & Capron, 1996). Furthermore, the number of eosinophils has a direct correlation with the severity of the late asthmatic reaction (Bousquet et al., 1990).

Interleukin-5 (IL-5) is a colony stimulating factor for the eosinophil lineage (Clutterbuck & Sanderson, 1988; Yamaguchi et al., 1988b), which also supports the viability of mature cells (Yamaguchi et al., 1988a) by suppressing activation of the caspase cell death cascade (Zangrilli et al., 2000). In addition, IL-5 enhances cellular activity and degranulation (Lopez et al., 1988), and is capable of amplifying chemotactic signals resulting in the recruitment of eosinophils to tissues (Foster et al., 2002). A pivotal role for IL-5 in chronic allergic inflammation has been confirmed by the capability of neutralizing anti-IL-5 monoclonal antibody (mAb) to inhibit antigen- and virus-induced airway hyperresponsiveness and eosinophil infiltration in the airways of mice (Nagai et al., 1993; Kung et al., 1995), guinea pigs (Chand et al., 1992; Gulbenkian et al., 1992; van Oosterhout et al., 1993a; van Oosterhout et al., 1993b) and primates (Egan et al., 1995; Mauser et al., 1995).

Although IL-5 possesses only a weak chemotactic activity for eosinophils (Wang et al., 1989; Yamaguchi et al., 1988b), it has been shown that upregulation of adhesion molecules occurs following IL-5 stimulation of circulating eosinophils resulting in increased adhesion to endothelial cells (Walsh et al., 1990). In a primate model for asthma, antibody against intracellular adhesion molecule-1 (ICAM-1) inhibited the adhesion of eosinophils to endothelial cells, decreasing eosinophil migration to the
lungs and significantly reducing the asthmatic reaction (Wegner et al., 1990). IL-5 is also able to induce a modest increase in the mRNA level of eotaxin, a chemoattractant specific for eosinophils that are known to play a role in helminth and allergic responses (Han et al., 1999).

The importance of eosinophilia in various eosinophilic diseases and the central role of IL-5 in controlling eosinophil numbers make this cytokine a primary target for therapeutic intervention. Several approaches may be taken to inhibit IL-5 production, which will be discussed further in Section 1.5.

1.2 Discovery of IL-5

1.2.1 Discovery of Murine IL-5

In the early 1970s, a number of different factors were described which showed activity on mouse B-cells in vitro. These preparations were mixtures of cytokines and the assays available did not distinguish between the molecules. It was not until the early 1980s that a group of high-molecular-weight activities emerged, although the identity of the activities was not appreciated in published reviews (Vitetta et al., 1984; Howard, Nakanishi & Paul, 1984). Three groups were working simultaneously to identify this activity: Swain and colleagues in California, who used the term B-cell growth factor II (BCGFII) (Swain et al., 1988); Vitetta in Texas, who called it B-cell differentiation factor μ (BCDFμ) (Vitetta et al., 1984); and Takatsu in Japan who characterized the same molecule as T-cell replacing factor (TRF) (Harada et al., 1985; Takatsu et al., 1988). This group, eventually, purified TRF and showed it was identical to BCGFII (Harada et al., 1985).

During the same period, Metcalf and colleagues, who were studying the colony stimulating factors (CSFs), demonstrated the production of eosinophilic colonies from mouse bone marrow in the presence of crude spleen-cell-conditioned medium (Metcalf et al., 1974). It is now clear that this medium contained murine IL-5 (mIL-5) because it was shown to produce a selective stimulation of human eosinophil
colonies (Metcalf, Cutler & Nicola, 1983) and IL-5 is the only eosinophil haematopoietic growth factor which demonstrates cross-species reactivity.

To identify the factor controlling eosinophil production, Sanderson and colleagues developed a liquid bone marrow culture system (Warren & Sanderson, 1985; Sanderson, Warren & Strath, 1985) in which the number of eosinophils is estimated directly by morphology, or indirectly by assay for eosinophil peroxidase. Using these methods, they showed that activated T-cells produced a factor which they named the eosinophil differentiation factor (EDF). In addition to activated T-cells, EL-4 mouse lymphoma cells also produced EDF. Unfortunately, these cells also synthesize a number of other cytokines, making purification of EDF difficult. To overcome this problem, Sanderson and colleagues created T-cell hybrids (Warren & Sanderson, 1985) that produced EDF and none of the other cytokines. One of these cell lines, NIMP-TH1, became a valuable source of EDF for purification and gene cloning.

The two lines of research came together in 1986, when EDF was purified and shown to be identical to BCGFII (Sanderson et al., 1986). It is interesting to note that the observations on the identity of TRF, BCGFII and EDF were made before the cloning of IL-5, which confirmed the biochemical data.

1.2.2 Discovery of Human IL-5

Soon after the discovery of mIL-5, Azuma and colleagues cloned an 816bp human IL-5 cDNA from a human leukemic T-cell line, ATL-2, using mIL-5 cDNA as probe (Azuma et al., 1986). The cloned cDNA encoded the IL-5 precursor of 134 amino acids containing an N-terminal signal sequence.

In 1987, Tanabe and colleagues utilized the human IL-5 (hIL-5) cDNA to screen DNA phage libraries to further clone and characterize the chromosomal gene for hIL-5 (Tanabe et al., 1987). Phage libraries containing human foetal liver DNA, human placental DNA, and DNA of the human myeloma cell line 266B1 were screened with a fragment of the hIL-5 cDNA, which contained the 5'-untranslated region and the entire coding region, as probe (Tanabe et al., 1987). Restriction
Figure 1.1: Position of IL-5 and other cytokine genes on human chromosome 5.
enzyme cleavage mapping of positive DNA libraries, followed by Southern blot hybridisation showed that the DNA fragments contained the same 3.2kb BamHI fragment that hybridised with the hIL-5 cDNA. Through this method, it was determined that hIL-5 is encoded by a single copy gene comprising of 4 exons and 3 introns (Azuma et al., 1986).

Chromosomal mapping by in situ hybridisation determined the location of the hIL-5 gene to be on chromosome 5q23.3-31.1 (Takahashi et al., 1989). As the interleukin-4 (IL-4) (van Leeuwen et al., 1989), granulocyte-macrophage colony stimulating factor (GM-CSF) (Pettenati et al., 1987) and interleukin-3 (IL-3) (Le Beau et al., 1987) genes were previously mapped on human chromosome 5, further study using pulsed-field gel electrophoresis showed that these genes are tightly linked. The IL-3 and GM-CSF genes are only 9kb apart, whereas the IL-4 and IL-5 genes are approximately 100kb apart (van Leeuwen et al., 1989) (Fig. 1.1). Linkage of these genes, therefore, suggested important implications in the evolutionary origin and regulation of expression of these genes, which will be discussed further in Section 1.3.

1.2.2.1 Human IL-5 Protein

Determination of the three-dimensional structure of hIL-5 by X-ray crystallography (Milburn et al., 1993) revealed that IL-5 belongs to a family of structurally related proteins, which includes IL-2, IL-4, macrophage colony stimulating factor (M-CSF), GM-CSF and growth hormone. These proteins fold to produce a tertiary structure of four bundled α-helices (designated A, B, C and D from the N-terminus), arranged with an up-up, down-down topology (Milburn et al., 1993).

hIL-5 is a homodimeric glycoprotein consisting of two identical polypeptide chains 115 amino acid residues long (113 in mouse) (Fig. 1.2). An unusual structural feature of the homodimeric IL-5 is that it forms two four-helical bundles, each containing three helices (A-C) from one chain and one from the other (D). Each bundle contains helices A and D paired in an antiparallel orientation to form one face of the molecule, and the B and C helices, also paired in an antiparallel orientation, to form the other face. The AD and BC helical pairs of each bundle are at an angle of
Figure 1.2: The human IL-5 molecule adapted from Milburn et al. (1993). It consists of two identical polypeptide chains, shown here in different colors. Nt and Ct denote N-terminus and C-terminus of the chain, respectively. A-D and A’-D’ denote the helices that made up the polypeptide chains.
40° relative to each other, an unusual feature of four-helical bundle cytokines (Milburn et al., 1993).

The hIL-5 glycoprotein has a theoretical mass of 24 kDa, and the characterization of *Escherichia coli*-expressed recombinant hIL-5 gives a molecular mass of 26 kDa (Graber et al., 1993). However, recombinant hIL-5 expressed in mammalian systems has a molecular mass of over 45 kDa as estimated by SDS-PAGE analysis (Sanderson, Warren & Strath, 1985); thus, a large proportion of the mass is carbohydrate, added during intracellular processing (Tominaga et al., 1990).

1.2.2.2 Human IL-5 Receptor

The hIL-5 protein has also been shown to contain a functional nuclear localization sequence (NLS), which allows the hIL-5 receptor to translocate to the nucleus following hIL-5 binding (Lauder, McKenzie & Sanderson, 2003). Molecular cloning of receptor subunits and reconstitution experiments revealed that the IL-5, IL-3 and GM-CSF receptors are composed of a cytokine-specific α-subunit and a common β-subunit (Tavernier et al., 1991; Miyajima et al., 1993). The α-subunit is the major ligand binding subunit and on its own does not seem to induce any of the biological activities ascribed to GM-CSF, IL-3 and IL-5 in hemopoietic cells (Robb et al., 1995; Muto et al., 1995). The β-subunit, on the other hand, converts the ligand-bound α-subunit to a high affinity state, and is important for most of the signalling (Kitamura et al., 1991; Hayashida et al., 1990; Bagley et al., 1997).

IL-5, IL-3 and GM-CSF have been shown to be involved in the regulation of hemopoietic progenitor cells, suggesting common activities between these cytokines. Despite this, only IL-5 has been shown to be a contributing factor of eosinophilia. Thus, the functional overlap among the genes can be explained by this shared receptor subunit, whereas the cytokine-specific functions may be explained by the differential intracellular signalling events originating from the cytokine specific α-subunit (Miyajima et al., 1992). These signalling events would be discussed further in Section 1.3.3.
1.2.2.3 Human IL-5 Gene

IL-5 may be regarded as belonging to the IL-4 gene family consisting of the IL-4, IL-13, IL-3 and GM-CSF genes. They are part of the 5q31 cytokine gene cluster located on chromosome 5 in human (Campbell et al., 1987) (Fig. 1.1) and the syntenic region of chromosome 11 in the mouse (van Leeuwen et al., 1989). Although there is no overall sequence homology at either the nucleotide or amino acid level between the cytokine genes, the localization and gene structural similarities suggest a common evolutionary origin (Sanderson, 1992). The IL-5, IL-4 and GM-CSF genes have three introns at roughly similar positions of the polypeptides (Tanabe et al., 1987). The IL-3 gene has four introns, and the relative locations of the first, second, and fourth introns resemble those of the three introns of the IL-5, IL-4 and GM-CSF genes, indicating that the latter genes may have lost one intron during evolution (van Leeuwen et al., 1989).

The exons and introns of both mIL-5 and hIL-5 genes show areas of similarity between the mIL-5 and hIL-5 sequences (Fig. 1.3). However, the murine sequence includes a 738bp fragment (Alu-like repeat) in the 3′-untranslated region, which is not present in the human sequence (Sanderson, 1998), making the murine transcript longer than the human transcript (1.6kb and 0.9kb, respectively) (Campbell et al., 1988).

1.3 Expression of the hIL-5 Gene

1.3.1 Sources of IL-5

The characterization, purification and cloning of mIL-5 employed T-cell lines or lymphomas as the source of material, suggesting that T-cells are an important source of the cytokine. In a study of IL-5-producing cells in bronchial biopsies from asthmatic subjects, it was concluded that T-cells are the major cellular source of IL-5 (Ying et al., 1997). The apparent dominance of mast cells in some studies was attributed to the fact that mast cells store IL-5 in their granules, whereas T-cells secrete IL-5 rapidly as it is synthesized. Thus, immunohistological staining for IL-5 underrepresents the number of T-cells compared with in situ hybridization.
Figure 1.3: Maps of the A) human and B) murine IL-5 genes. The solid horizontal lines indicate introns with significant homology between the human and murine sequences. Dotted lines represent introns with very little sequence conservation, while exons are represented as purple boxes and contain the codons for exact number of amino acids. Yellow boxes represent untranslated regions, while the grey box represents the Alu-like repetitive sequence in the murine gene that is absent from the human gene.
The demonstration that IL-5 as well as other cytokine mRNAs are produced by human (Barata et al., 1998) and mouse (Csonga et al., 1998) mast cell lines raises the possibility that these cells may serve to induce or amplify the development of eosinophilia. Similarly, the observation that human Epstein-Barr virus-transformed B-cells produce IL-5 raises the possibility that B-cells may be an additional source of this cytokine (Paul et al., 1990). Furthermore, eosinophils themselves have been demonstrated to produce IL-5, although they do not appear to produce enough to sustain their own survival (Bao et al., 1996; Broide, Paine & Firestein, 1992). Other sources of hIL-5 include natural killer (NK) cells (Warren et al., 1995), basophils (Ying et al., 1995), bone marrow microvascular endothelial cells (Mohle et al., 1997) and epithelial cells (Salvi et al., 1999).

1.3.2 Co-Expression\(^a\) of hIL-5 with Other Cytokines

The cytokines belonging to the IL-4 gene family (Section 1.2.2.2) are produced by activated lymphocytes, primarily those belonging to the T-helper type 0 (Th0) and type 2 (Th2) subsets of mature CD4\(^+\) T-helper cells (Abbas, Murphy & Sher, 1996; Mosmann & Coffman, 1989). IL-5 is often co-expressed with IL-4 and IL-13 (Shimbara et al., 2000; Verheyen et al., 2000), allowing speculation that these genes may be co-ordinately regulated\(^b\) (Loots et al., 2000; Takemoto et al., 2000).

While members of the IL-4 gene family show similarities of protein and gene structures, and have overlapping expression, many studies have also indicated that different specific signals are required for the induction of each of these genes. In mouse T-cells, treatment with IL-2 induced IL-5 and IL-13 mRNA expression but did not induce detectable amounts of IL-4 or GM-CSF mRNA (Bohjanen, Okajima & Hodes, 1990; Verheyen et al., 2000). Furthermore, IL-4 appeared to increase the IL-2-induced production of IL-5 and IL-13, while IL-12 suppressed it.

\(^a\) Co-expression denotes the simultaneous expression of genes in an organism, where it may not necessarily happen in the same cells, nor due to coordinate regulation of the genes.

\(^b\) Co-ordinate regulation denotes the simultaneous gene expression, controlled at the genetic level, in the same cell.
In humans, a Th2-like pattern of cytokine mRNA expression has been demonstrated in asthmatic patients. T-cells purified from peripheral blood of non-atopic asthmatics secreted increased amounts of IL-5 but not IL-4 (Robinson et al., 1993), whereas those from atopic patients secreted elevated quantities of both IL-4 and IL-5 (Corrigan & Kay, 1992). These differences are further emphasized by the observation that pertussis toxin induces IL-4 but not IL-5 mRNA synthesis, whereas cyclophosphamide stimulates the transcription of IL-5 but not of IL-4 (Sewell & Mu, 1996).

In another study, the utilization of SB203580, a pyridinyl imidazole derivative that inhibits the actions of p38 MAP kinase, showed a selective effect on IL-5 synthesis (Mori et al., 1999a). Human T-cell clones were pre-stimulated by anti-CD3 antibody to release Interferon-γ (IFN-γ), IL-2, IL-4 and IL-5 into the culture supernatants; however, the addition of SB203580 specifically inhibited production of IL-5. From these observations, it appears that IL-4 and IL-5 expression are not co-ordinately regulated, suggesting that unique control mechanisms for these lymphokines do exist.

1.3.3 Pathways Leading to the Activation of the hIL-5 Gene

The dependence of eosinophil responses on T-lymphocytes described in the 1970s (Basten & Beeson, 1970), and cloning of IL-5 as a T-cell-derived eosinophil-specific cytokine provided a link between T-cell activation and eosinophilic disease (Kinashi et al., 1986; Campbell et al., 1987). Activation of T-cells requires the interaction of the T-cell receptor (TCR) with antigen in association with the major histocompatibility complex (MHC), which leads to an increase of intracellular calcium concentration and activation of protein kinase C (PKC) (Perlmutter et al., 1993). However, stimulation of the TCR alone is insufficient to fully activate T-cells; a second signal generated by the CD28 ligand (Jenkins & Johnson, 1993) and provided by antigen-presenting cells (APC) (Rincon & Flavell, 1994) is also required.

Several combinations of regulatory molecules have been shown to best induce IL-5 synthesis in vitro. α-CD28 antibody (Karlen, D'Ercole & Sanderson, 1996) or cyclic-adenosine-monophosphate (cAMP) (Gruart-Gouilleux, Engels & Sullivan, 1995; Lee
et al., 1993), in combination with phorbol-12-myristate-13-acetate (PMA), was shown to be necessary for optimal induction of IL-5 synthesis. In EL4 mouse lymphoma cells, PMA/α-CD28 stimulation activates expression of a variety of cytokine genes including IL-2, IL-3, IL-4, IL-10 and GM-CSF (Abbas, Murphy & Sher, 1996). The α-CD28 pathway is resistant to cyclosporin A (CsA) (Lai & Tan, 1994); however, the production of IL-5, as many other cytokines, is sensitive to CsA (Mori et al., 1994; Okudaira et al., 1995). Similar results were also obtained in a human T-cell line PER117, whereby PMA-induced expression of IL-5 was further enhanced by either cAMP or α-CD28 antibody (Mordvinov et al., 1999b). These results suggest an alternative pathway for the induction of the IL-5 gene exists, one that involves the Ca\(^{2+}\)-dependent calcineurin phosphatase whose activity is suppressed by CsA (Schwaninger et al., 1995).

While cAMP, acting through the protein kinase A (PKA) pathway, markedly enhances the effect of PMA on the induction of IL-5, it has an inhibitory effect on IL-2, IL-3, IL-10 and GM-CSF (Chen & Rothenberg, 1994; Lee et al., 1993), and no effect on IL-4 (McCaffrey et al., 1993). This suggests that IL-5 expression is controlled by at least two independent co-stimulatory pathways. One of these is the CD28 pathway, which may be regarded as a common pathway for activation of cytokine genes. The other is the cAMP-dependent pathway that, in context of T-cell cytokine genes, appears to be unique for induction of IL-5 expression.

Other regulatory molecules such as the lectin concanavalin A (ConA), histamine, IL-1α, forskolin and monoclonal antibodies to CD2 and CD45 are all able to induce IL-5 expression (Naora & Young, 1994; Lee et al., 1998; Schmidt et al., 1994). In addition, the binding of certain cytokines to their receptors can also influence the expression of the IL-5 gene. IL-2, for example, has been shown to upregulate IL-5 production in T-cells (Kuiper et al., 1994). Likewise, IL-4 can increase IL-5 production in both T-cells and NK cells (Warren et al., 1995).
1.4 Transcriptional Control of the hIL-5 Gene

Generally, a DNA sequence called the core promoter is located immediately adjacent to and upstream of the gene. The core promoter binds and directs RNA polymerase II to begin transcribing at the correct start site. The transcriptional start site of the hIL-5 gene was determined through a sequence comparison of human cDNA to murine cDNA by Azuma and colleagues (Azuma et al., 1986).

Immediately upstream of the core promoter is a regulatory promoter, and further away, either upstream or downstream of the gene, there may be enhancer elements (Carey & Smale, 2000). The following sections will describe the regulatory elements of the hIL-5 gene.

1.4.1 Positive Regulatory Elements (Activators)

A typical core promoter encompasses DNA sequences between -40 to +50 relative to a transcription start site (Smale, 1994). It alone is generally inactive in vivo, but in vitro can bind to the general machinery and support low or ‘basal’ levels of transcription. Activators then greatly stimulate transcription levels, and the effect is called activated transcription (Carey & Smale, 2000). The binding of sequence-specific DNA-binding proteins, called activators and co-activators, to regulatory promoters and enhancers, controls transcriptional regulation (Blackwood & Kadonaga, 1998).

The expression of hIL-5 appears to be predominantly regulated at a transcriptional level (Naora & Young, 1994), and the conservation between species of the proximal region of the IL-5 promoter is indicative of the importance of regulatory elements present within that region (Fig. 1.4). The 5'-flanking region of the IL-5 RNA initiation site contains the TATA-box and additional motifs involved in transcription of the gene (Fig. 1.5). Immediately upstream of the TATA-box, located between nucleotides -56 and -42 (Karlen, Mordvinov & Sanderson, 1996) is a short sequence called the Conserved Lymphokine Element 0 (CLE0) (Table 1.1). Mutations introduced within this element abolish IL-5 expression, indicating that this sequence is essential for promoter activity (Naora et al., 1994; Thomas, Mordvinov & Sanderson, 1999; Gruart-Gouilleux, Engels & Sullivan, 1995).
CLE0-like elements are also found to exist within the regulatory regions of several other cytokine genes, including IL-3 (Shoemaker, Hromas & Kaushansky, 1990), IL-4 (de Boer et al., 1999) and GM-CSF (Masuda et al., 1993) (Table 1.1). The CLE0-like element of the IL-4 gene contains consensus binding sequences for both AP1 and NF-AT; yet its presence produces only a basal promoter activity (Abe et al., 1992), indicating that it may be inactive. The IL-3 CLE0-like element appears to serve a dual role to relieve the action of the inhibitor region (located between −173 and −61) and enhance IL-3 expression (Mathey-Prevot et al., 1990). However, it also appears to be a very weak activator by itself, and might require the binding of repressor proteins onto the repressor region located between −271 and −250.

The CLE0 element present in the human GM-CSF gene showed the most homology to the IL-5 CLE0 element (Table 1.1), differing only by an A→T substitution. Similar to IL-5 CLE0, the GM-CSF CLE0 is located near the transcription initiation site, and is required for GM-CSF promoter activity in T-cells (Nimer et al., 1990). Furthermore, mutations within the element eliminated basal and inducible promoter activity (Fraser et al., 1994; Nimer et al., 1990). In addition, the AP1 site within the GM-CSF CLE0 alone is insufficient for enhancer activity in T-cells, and it was demonstrated that the Ets transcription factor-binding site at the 3′-end of the element is required for full enhancer activity (Nimer et al., 1996).

IL-5 CLE0 contains sequences similar to the binding sequences for AP1 and NFAT, but only the AP1 moiety has been demonstrated to be important for inducible complex formation in EL4 cells (Siegel et al., 1995; Karlen, D'Ercole & Sanderson, 1996). The AP1 members that bind to IL-5 CLE0 in an inducible fashion have recently been identified as JunD and Fra-2 in the human leukemic T-cell line PER117 (Thomas, Mordvinov & Sanderson, 1999). The same report also identified constitutive binding of Oct-1 and inducible binding of Oct-2 to hIL-5 CLE0 (Fig. 1.5).
Figure 1.4: The conservation of the 500bp region of the IL-5 promoter between species. Regions of homology are highlighted in yellow, and IL-5 regulatory elements within the proximal region are presented in bold-type.
**Figure 1.5**: Transcription factors binding IL-5 proximal promoter in A) resting and B) activated T-cells.
Table 1.1: CLE0-like elements found within regulatory sequences of cytokine genes found in the 5q31 gene cluster of chromosome 5. Putative AP-1 binding site is underlined. Sequences within the CLE0 of each gene homologous to the AP-1 consensus (5'-TGA(G/C)TCA-3') are indicated in **bold**.

<table>
<thead>
<tr>
<th>Cytokine Gene (human)</th>
<th>5'-Sequence-3'</th>
<th>Position</th>
<th>Binding Transcription Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>ATTATTCATTTCCCT</td>
<td>-56 to -42</td>
<td>AP-1 (Fra2 and JunD), Oct1 and Oct2 (Thomas, Mordvinov &amp; Sanderson, 1999)</td>
</tr>
<tr>
<td>IL-4</td>
<td>TAAACTCATTTCCCCT</td>
<td>-64 to -49 (de Boer et al., 1999)</td>
<td>AP-1 (JunB and JunD) (Hodge, Rooney &amp; Glimcher, 1995), and NF-AT (Li-Weber, Giasi &amp; Krammer, 1998)</td>
</tr>
<tr>
<td>IL-3</td>
<td>TGAATCAGGCTTCCCCT</td>
<td>-301 to -285 (Shoemaker, Hromas &amp; Kaushansky, 1990)</td>
<td>AP-1 (c-Fos, c-Jun, JunD and JunB) (Shoemaker, Hromas &amp; Kaushansky, 1990)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ATTAATCATTTCCCT</td>
<td>-42 to -29 (Nimer et al., 1996)</td>
<td>AP-1 (c-Fos and c-Jun) and NF-AT (Masuda et al., 1993), and Ets1 (Thomas et al., 1997)</td>
</tr>
</tbody>
</table>
The importance of IL-5 CLE0, however, has been questioned in several studies. In one study, deletion of the hIL-5 promoter to position -80 increased constitutive expression and removed inducibility in mouse D10 cells (Stranick et al., 1997). This led the authors to conclude that CLE0 is not responsible for inducible expression, an observation also made independently by Tsuruta and colleagues in Japan (Tsuruta et al., 1995) and Prieschl and colleagues in Austria (Prieschl et al., 1995). Another study by Stranick and colleagues showed that mutations in the CLE0 element had little effect on reporter expression (Stranick et al., 1995), which lead to the conclusion that IL-5 CLE0 was of little importance to the expression of the gene. This thesis will, therefore, address the question of whether IL-5 CLE0 is of any importance in the regulation of IL-5 gene expression (see Section 1.5).

IL-5 CLE0 also works in concert with other activation elements in the IL-5 promoter. The binding of Oct (Gruart-Gouilleux, Engels & Sullivan, 1995) and GATA (Siegel et al., 1995; Lee et al., 1998; Yamagata et al., 1995; Zhang et al., 1997) proteins at two sites immediately upstream of the CLE0 element has been reported. Binding of proteins to the Oct sequence was dependent on cell activation, whereas binding of GATA-3 was constitutive (Fig. 1.5). Mutations in the GATA(-70) element have also been shown to abolish IL-5 expression (Lee et al., 1998; Zhang et al., 1997). Additionally, in mouse cell lines, the GATA-3 transcription factor was shown to transactivate IL-5 but not IL-4 gene promoters, whereas the c-Maf transcription factor transactivated IL-4 and not IL-5 gene promoters (Ferber et al., 1999; Kishikawa et al., 2001; Lee et al., 2000).

NF-AT factors, in conjunction with AP1 family members, has also been found on the mIL-5 P-sequence located at positions -117 to -92 in EL4 cells (Lee et al., 1995), and to a similar position of the hIL-5 promoter in human T-cell clone SP-B21 (Stranick et al., 1997). The role of this site in IL-5 gene expression remains controversial as mutation analysis in some studies suggested a critical role for this site (Stranick et al., 1997; Lee et al., 1995; Lee et al., 1998), while others have shown little or no effect (Siegel et al., 1995; Zhang et al., 1997). This NF-AT site, however, has recently been shown to be involved in positive regulation of hIL-5 in both PER117 cells (Fig. 1.5) and peripheral blood lymphocytes (de Boer et al., 1999).
In 1995, Gruart-Bouilleux and colleagues reported the presence of a positive regulatory element between positions -244 and -237 (Fig. 1.5). The regulatory element, containing an Octamer motif, was found to bind factors antigenically related to Octamer factors and when mutations were introduced in the motif, promoter activity was reduced drastically.

1.4.2 Negative Regulatory Elements (Repressors)

Gene expression can also be regulated by repression mechanisms through repressor and co-repressor proteins. In general, transcriptional repression can be divided into three categories. First, repression can occur by the inactivation of an activator. Secondly, proteins that inhibit the formation of a pre-initiation complex can mediate gene repression. Third, transcription repression is mediated by a specific DNA element and DNA-binding protein, which act to repress the transcription of a gene (Herschbach & Johnson, 1993; Hanna-Rose & Hansen, 1996).

Two palindromic elements analogous to the murine IL-5 palindromic elements (Schwenger et al., 1998) have also been identified in the hIL-5 promoter (Fig. 1.5), and are called human palindromic regulatory element 1 (hPRE1-IL-5) and 2 (hPRE2-IL-5). The hPRE1-IL-5 element is located between nucleotides -78 to -94 (Schwenger & Sanderson, 1998), and by binding transcription factors Oct1 and YY1, appears to be involved in suppression of IL-5 transcription in human T-cells (Mordvinov et al., 1999b). Furthermore, this element is also conserved among IL-5 genes of different species (Fig. 1.4); the homologous murine palindromic element (mPRE) 1 is also located at positions -79 to -90 of the mIL-5 promoter. In contrast to hPRE1, however, mPRE1 is involved in positive induction of the mIL-5 gene (Schwenger et al., 1998). The hPRE2-IL-5 element is located upstream of hPRE1-IL-5 (between nucleotides -452 and -462), and by binding both YY1 and NF-AT factors, it is reported to act as a repressor element (Schwenger et al., 1999).

A negative regulatory element identified at position -404 to -395 of the hIL-5 promoter was found to bind the GATA-3 factor in PER117 cells (Schwenger et al., 2001). Mutations of this site resulted in an up-regulation of hIL-5 gene expression in unstimulated cells. Therefore, it was suggested that the -400 GATA-3 binding site
suppresses hIL-5 expression by maintaining a specific promoter structure that prevents un-induced expression (Schwenger et al., 2001).

Other negative regulatory elements have also been identified in the hIL-5 promoter. Investigations of the promoter in mouse T-cells has demonstrated that two negative regulatory elements lie between positions –404 to –312 (Gruart-Gouilleux, Engels & Sullivan, 1995) and –172 to –127 (Stranick et al., 1997). The activity of these elements is dependent on activation of the cells since deletion of the regions results in a marked increase in inducible promoter activity. Nuclear proteins that may interact with these elements, however, have not yet been characterized.

1.4.3 Post-transcriptional Regulation of the hIL-5 Gene

Regulatory elements are generally known to be present on the 5'-flanking region of a gene, such as those described above, yet they have also been described in the untranslated regions (UTR). The majority of these elements function as post-transcriptional regulatory elements, influencing mRNA stability (Shaw & Kamen, 1986; Koeffler, Gasson & Tobler, 1988; Elias & Lentz, 1990; Guba et al., 1989) or translational efficiency (Kruys et al., 1987; Kruys & Huez, 1994). While post-transcriptional regulation is emerging as an important control point in cytokine gene expression, no evidence was found to suggest that hIL-5 is regulated at this level by the 3'-UTR (Thomas et al., 1999).

1.5 IL-5 Inhibition

1.5.1 IL-5 and Diseases

hIL-5 is a cytokine that is not encountered at high levels in healthy individuals (Sanderson, 1989). As described previously (Section 1.3), the IL-5 protein is predominantly produced by activated CD4 T-lymphocytes (Okudaira et al., 1995; Till et al., 1997) and eosinophils (Barata et al., 1998; Bao et al., 1996), and is, therefore, a part of the immune response that leads to cell-mediated immunity, and a series of disorders.
1.5.1.1 Parasitic Infections

In many helminth infections, the number of eosinophils increases dramatically, often without any concurrent increase in the number of other leukocytes, so that eosinophils become the dominant cell type. Helminth-induced airway hyperresponsiveness is associated with eosinophilia, but can be distinguished from asthma by the positive effects of anti-helminth treatment. In helminth-infected individuals, a circulating population of IL-4- and IL-5-secreting cells is expanded following exposure to parasite-derived antigen (Mahanty et al., 1993), which correlated with the acuteness of the disease.

The severities of other parasitic infections, such as alveolar echinococcosis of the liver (Sturm et al., 1995) and infection by *Paragonimiasis westermani* (Taniguchi et al., 2001) have also been shown to correlate with the IL-5 levels in the circulating blood. Alveolar echinococcosis is a life-threatening disease caused by *Echinococcus multilocularis*, and peripheral blood mononuclear cells stimulated by its antigen have enhanced expression of Th2-like cytokines, especially IL-5 (Sturm et al., 1995). Infection by *Paragonimiasis westermani* is a parasitic disease typically associated with eosinophilia and eosinophilic pleural effusions, where the levels of eosinophils in peripheral blood and pleural fluid correlate with high concentrations of IL-5 (Ashitani, Kumamoto & Matsukura, 2000; Taniguchi et al., 2001). The induction of IL-5 expression indicates a critical role of this cytokine in these diseases.

1.5.1.2 Allergic Diseases

Asthma is a highly prevalent respiratory disease involving bronchial inflammation. During the last 30 years, there has been a considerable increase in the incidence of this disease all around the world (Miescher & Vogel, 2002). It is a disease in which cell-mediated immunity orchestrates the accumulation of neutrophils, T-lymphocytes and, more importantly, eosinophils (Lemanske & Busse, 2003). There is much evidence that the release of eosinophil major basic protein and eosinophil-derived neurotoxin (indicators of local degranulation) at the sites of injury are responsible for the bronchial epithelial damage within the lungs of asthmatics (Gleich, 1990; Gleich et al., 1988).
Studies with IL-5 monoclonal antibodies clearly support a role for IL-5 in eosinophilia and asthma. Pre-treatment with anti-IL-5 monoclonal antibodies can suppress allergen induced airway eosinophilia (Viola et al., 1998; Chand et al., 1992; Coffman et al., 1989). In IL-5 knock-out mice, both allergen induced eosinophilia and airway hyperresponsiveness are abolished (Foster et al., 1996). Furthermore, in OVA-sensitised and challenged mice, treatment with anti-IL-5 antibodies did not affect the function of B-cells, T-cells, or mast cells, but rather, completely inhibited the development of eosinophilic lung inflammation and airway hyperresponsiveness (Hamelmann et al., 1999).

In patients suffering from allergic rhinitis, IL-5 gene expression is more significantly increased than in patients with a stable disease (Lee et al., 1997). The presence of immunoglobulin E (IgE) and eosinophil cationic protein (ECP), a marker of eosinophil activation, is also increased in patients with this disease (Kramer et al., 2000).

Chronic atopic dermatitis lesions are also associated with increased activation of the IL-5 gene, and the maintenance of chronic inflammation is predominantly associated with increased IL-5 expression and eosinophil infiltration. The clinical severity of this disease is significantly reduced as IL-5 level is decreased (Kondo et al., 2000).

### 1.5.1.3 Other Diseases

Idiopathic hyper eosinophilic syndrome (HES) is a disease characterized by unexplained hypereosinophilia persisting for at least 6 months and leading to organ damage (Bain, 2000). The diagnosis is based on the exclusion of other causes of eosinophilia. A causative mechanism of this disease is IL-5 secretion by a clone of T-cells (Cogan et al., 1994) or by phenotypically aberrant polyclonal T-cells (Bank et al., 1998). No recent advances have occurred in the therapy of HES, and hydroxyurea, glucocorticoids, and interferon remain the most commonly used therapeutic agents (Bain, 2000).

IL-5 and eosinophilia have also been associated with diseases such as chronic obstructive pulmonary disease (Matsui, Tanaka & Nishikawa, 2001), acute eosinophilic pneumonitis (Taniguchi et al., 2000), primary biliary cirrhosis (McGill
et al., 2001), and Wells’ syndrome (eosinophilic cellulitis) (Espana et al., 1999). Patients suffering from these diseases have increased IL-5 levels in their peripheral blood (Matsui, Tanaka & Nishikawa, 2001; Espana et al., 1999) and/or BAL fluid (Taniguchi et al., 1999; Okubo et al., 1998).

1.5.2 hIL-5 Inhibition

The production of eosinophils from progenitor cells in the bone marrow is under the exclusive control of IL-5 (Clutterbuck, Hirst & Sanderson, 1989). IL-5 also enhances the release of eosinophils from the marrow. Taken together, these two effects result in a rapid and sustained increase in eosinophils in the circulation. In tissues, IL-5 acts as a potent eosinophil survival factor, preventing the apoptotic cell death that usually results in the rapid clearance of cells (Yamaguchi et al., 1991). In view of this spectrum of activity, and the range of diseases aggravated by IL-5, it is clear that modulating the activity of IL-5 will have a major impact on many aspects of eosinophil function, leading to clinical benefits in IL-5-induced diseases.

1.5.2.1 Antibodies

One of the approaches used in reducing the level of IL-5-induced eosinophilia is to use antibodies directed against IL-5 or IL-5 receptor (IL-5R). In man, the IL-5R is found primarily on eosinophils (Sanderson, Warren & Strath, 1985; Greenfeder et al., 2001; Lopez et al., 1992) and basophils (Yamada et al., 1998). IL-5Rα-deficient (IL-5Rα−/−) mice were shown to have a decreased number of B-cells and peripheral eosinophils. Furthermore, B-cells and eosinophil precursors from these mice do not proliferate or differentiate in vitro in response to IL-5 (Yoshida et al., 1996). Thus, the interaction between IL-5 and its receptor is required to regulate the development of B-cells in mouse and eosinophils in human. A single injection of anti-IL-5R antibody reduced peripheral blood eosinophils in eosinophilic transgenic mice to control levels (Hitoshi et al., 1991). However, it is unclear whether the antibody acts by blocking IL-5 binding to its receptor, or whether cells expressing the receptor are eliminated by immune mechanisms.

The development of an antibody to the IL-5, which could block IL-5 binding to the receptor, would prevent the signalling of proliferation, maturation and degranulation
of eosinophils. When mice infected with *Nippostrongylus brasiliensis* were treated with an anti-IL-5 antibody, no eosinophils were observed and the number of eosinophil precursors in the bone marrow was also reduced (Coffman et al., 1989). Similar results were observed in mice infected with *Schistosoma mansoni* (Sher et al., 1990), the nematodes *Heligmosomoides polygyrus* (Urban et al., 1991) and *Strongyloides venezuelensis* (Korenaga et al., 1991) following anti-IL-5 treatment. More importantly, as the antibody did not block the effect of IL-3 or GM-CSF, these experiments demonstrated the essential role of IL-5 in the control of eosinophilia.

1.5.2.2 Antisense Oligonucleotides

Another approach in reducing IL-5 level is the administration of antisense oligonucleotides to block transcription of specific regulatory factors thus affecting gene transcription, or to block transcription of the gene itself. Use of these compounds in human and mouse cells have specifically inhibited IL-5 production (Weltman & Karim, 1998; Karras et al., 2000). A 16-mer phosphorothioate-modified oligo with high GC content was shown to totally inhibit IL-5 production from peripheral blood mononuclear cells (PBMC) (Weltman & Karim, 1998), and a methoxyethylribose-modified phophorothioate 20-mer oligo was shown to specifically inhibit IL-5 production in mouse EL-4 cell line (Karras et al., 2000). This antisense technology has also been used to block the transcription factor GATA-3, and inhibit the development of allergic airway inflammation in sensitised mice (Finotto et al., 2001). The use of full-length antisense GATA-3 cDNA has also been shown to inhibit IL-5 gene expression in mouse D10 cells (Zhang, Yang & Ray, 1998; Zheng & Flavell, 1997).

1.5.2.3 hIL-5 Transcriptional Inhibitors

hIL-5 gene expression can be positively influenced by several cytokines (Section 1.3). There are also cytokines that have suppressive effects - both recombinant and endogenous IL-10 were shown to inhibit IL-5 mRNA expression in human T-cells, depending on the type of cell activation (Schandene et al., 1994; Staples et al., 2000). IL-12 and IFN-α have also been shown to inhibit IL-5 production in mice and human T-cells, respectively (Gavett et al., 1995; Schandene et al., 1996; Warren et al., 1995).
Macrolide antibiotics, such as cyclosporin A (CsA) and FK506, have also been used to inhibit IL-5 production (Rolfe, Valentine & Sewell, 1997; Mori et al., 1994). These compounds have been shown to strongly inhibit IL-5 mRNA transcription in cells stimulated with phytohemagglutinin (PHA), phorbol-12-myristate-13-acetate (PMA), and/or ionomycin (Andersson et al., 1992; Rolfe, Valentine & Sewell, 1997). Upon entry into the cells, these compounds form complexes with immunophilins, which subsequently bind to calcineurin, inhibiting its phosphatase activity. This, in turn, prevents the dephosphorylation and nuclear translocation of the cytoplasmic subunit of the NF-AT, rendering it incompetent (Nelson & Ballow, 2003). Recently, it was also shown that non-actin, a macrolide compound produced by Streptomyces griseus (Mori et al., 2000a), suppresses IL-5 synthesis by human T-cells without affecting IL-2 or IL-4 (Mori et al., 2001).

IL-5 expression also depends on de novo protein synthesis, whereby at least one protein, critical for the induction of IL-5 gene transcription, is newly synthesized in response to T-cell stimulation (Schwenger et al., 2002). Protein synthesis inhibitors cycloheximide (CHX) and anisomycin completely inhibited IL-5 mRNA synthesis in primary T-cells and murine Th2 cell clone D10.G4.1; however, they did not inhibit the expression of IL-2, IL-3, IL-4, IL-10 and GM-CSF mRNAs (Naora & Young, 1995; Stranick et al., 1995; van Straaten et al., 1994). Similar results were also obtained in human T-cell line PER117, where CHX completely inhibits IL-5 but not IL-4 mRNA (Schwenger et al., 2002).

An orally active inhibitor of IL-5 mRNA expression and protein production, R146225, has also been shown to be a potent inhibitor, irrespective of the cell system or stimulating agent (van Wauwe et al., 2000). R146225 is a novel six-substituted azauracil derivative that abrogated IL-5 mRNA levels, but has little or no effect on IL-2 and IL-4, and enhances the production of IFN-γ.

1.5.2.4 Glucocorticoids
Glucocorticosteroids (GCS) remain the most effective anti-eosinophilic drugs available (Barnes, Pedersen & Busse, 1998). They are extremely potent, anti-inflammatory or immuno-suppressive hormones whose actions are mediated by a variety of mechanisms that alter cell numbers and function (Nelson & Ballow, 2003).
The anti-inflammatory effects of GCS result from the inhibition of transcription by direct binding of GCS to GCS response elements (GRE), or by the production of proteins that have inhibitory effects on target gene transcription (Burnstein & Cidlowski, 1989).

GCSs have been demonstrated to be exogenous modulators of IL-5 activity, downregulating IL-5 production. GCS treatment in patients with moderate asthma revealed a decrease in bronchial responsiveness, bronchial eosinophilia, and the number of bronchoalveolar lavage (BAL) cells expressing IL-5 mRNA (Robinson et al., 1993; Leung et al., 1995). In another study, GCS treatment was found to significantly decrease peripheral blood eosinophil counts and serum IL-5 (Corrigan et al., 1993).

The synthetic GCS dexamethasone has also been shown to inhibit IL-5 production from Th0, Th1, and Th2-like T-cell clones derived from BAL fluid, blood, and from allergen-specific and randomly cloned T-cells (Braun et al., 1997; Krouwels et al., 1996). These studies indicate the effectiveness of GCSs in downmodulating the expression of IL-5 in asthmatic patients. However, dexamethasone-induced IL-5 inhibition was found to be dependent on the type of cell stimulation. Rolfe and colleagues reported that dexamethasone could inhibit IL-5 gene expression in PBMC after treatment with PHA, PMA and recombinant human IL-2 (rhIL-2), but not ionomycin (Rolfe et al., 1992). This illustrates the different signalling pathways involved in IL-5 expression, and further studies are clearly needed to determine the efficiency of this drug in blocking the production of IL-5.

1.6 Aims of Thesis

1.6.1 Aims

Both GM-CSF CLE0 and IL-5 CLE0 elements are clearly important regulatory elements in their respective genes (Section 1.4.1.1). However, as mentioned, the importance of hIL-5 CLE0 has been questioned in several studies (Tsuruta et al.,
1995; Stranick et al., 1997; Prieschl et al., 1995). This work, therefore, aims to clarify the role of CLE0 in hIL-5 gene expression.

Furthermore, glucocorticoids inhibit both hIL-5 and mIL-5 production. The lack of glucocorticoid response elements (GREs) in the hIL-5 promoter suggests that glucocorticoids may act indirectly to inhibit hIL-5 gene expression. This thesis also aims to study the possible effect of dexamethasone, a synthetic glucocorticoid, on hIL-5 CLE0.

1.6.2 General Approach
A method commonly used in gene regulation studies is the transient expression of reporter constructs in a model cell line (Section 2.10). Reporter constructs containing the hIL-5 CLE0 element have been transiently transfected into cell lines to study the importance of hIL-5 CLE0 in hIL-5 expression. However, one of the main factors restricting studies of hIL-5 expression has been the limited availability of human T-cell lines that express IL-5 in an inducible fashion. In this study, PER117 and Jurkat cell lines were used to investigate the role of hIL-5 CLE0 in IL-5 gene regulation.

1.6.2.1 Reporter Constructs
To create the reporter gene constructs, DNA fragments or oligonucleotides containing the hIL-5 CLE0 or other hIL-5 proximal control elements were prepared and manipulated as described in Sections 2.3-2.5. More thorough descriptions of the specific treatments needed to create individual constructs could be found in the ‘Experimental Approach’ section of each chapter.

1.6.2.2 Cell Lines
As was mentioned, one of the main factors restricting studies of hIL-5 expression has been the limited availability of human T-cell lines that express IL-5 in an inducible fashion. Studies with primary T-cells is limiting because of transfection inefficiencies and difficulties in obtaining un-degraded nuclear extracts (Sanderson et al., 1999). Other tumorigenic cell lines have been used in IL-5 gene regulation.
studies, but they have disadvantages (Sanderson et al., 1999) that led to the use of PER117 cell line in this study.

PER117 cell line is a prothymocyte cell line established from bone marrow cells of an eighteen-month old baby with acute lymphoblastic leukaemia (Kees et al., 1994). This cell line was chosen as it inducibly expresses IL-5 and IL-4 and, as in primary T-cells, there is no detectable constitutive expression of IL-5 (Kees et al., 1994). Expression of IL-5 can be induced with PMA and further enhanced by cAMP, Cal and α-CD28 antibodies (Mordvinov et al., 1999a). Furthermore, IL-5 production can be inhibited by dexamethasone and CsA. These properties mimic the regulation of IL-5 observed in vivo, making this cell line a good model for our studies.

The Jurkat cell line is a T-cell leukaemia line that produces a variety of cytokines under mitogenic stimulation. The line used in this study was ATCC TIB-152 which inducibly expresses IL-4 but not IL-5 (Sanderson et al., 1999). These cells were used in this study to determine the extent of the effect of hIL-5 control elements in a cell line that does not normally express the gene.

The BAF-Luciferase cell line, originally established by this laboratory (Coombe et al., 1998), is an IL-3-dependent mouse cell line that has been stably transfected with the gene for the hIL-5Rα-chain. These cells will only grow in the presence of either murine or hIL-5. Furthermore, the cell line has also been stably transfected with a luciferase reporter gene under the control of a CMV promoter, which also acts as a marker of cell growth. These cells were used to measure the level of expression of the luciferase reporter gene used in this study.
CHAPTER 2

MATERIALS AND METHODS
2.1 Reagents and Enzymes

2.1.1 Reagents

- Acetate
- Acrylamide:Bis solution (29:1)
- Adenosine 5'-Triphosphate (ATP)
- Agar Bacteriological
- Agarose
- Ampicillin
- γ-32P-ATP
- Boric acid
- Bromophenol blue
- Calcium ionophore A23187 (Cal)
- Carbon dioxide (CO₂)
- α-Leu-CD28
- Dexamethasone
- Dibuteryl-cyclic-adenosine-monophosphate (cAMP)
- Dimethyl sulfoxide (DMSO)
- Distilled water (ddH₂O)
- Dithiothreitol (DTT)
- dNTPs
- Ethanol
- Ethidium bromide (C₂₁H₂₀BrN₃)
- Ethylene Glycol-bis-Aminoethyl-ether-tetra-acetic acid (EGTA)
- Ethylene-diamine-tetra-acetic acid (EDTA)
- Foetal calf serum (FCS)
- Formamide
- Glucose (C₆H₁₂O₆)
- L-Glutamine

BDH Chemicals, Australia
Bio-Rad Laboratories, USA
Sigma Chemicals, USA
Oxoid, England
Amresco, USA
CSL, Australia
Amersham, Australia
Pronalys, Selby Biolab, Australia
Sigma Chemicals, USA
Sigma Chemicals, USA
BOC Gases, Australia
Becton Dickinson, USA
Sigma Chemicals, USA
Sigma Chemicals, USA
BDH Chemicals, Australia
Baxter Healthcare, Australia
Progen, Australia
Boehringer Mannheim, Germany
Scot Scientific, Australia
Sigma Chemicals, USA
Sigma Chemicals, USA
BDH Chemicals, Australia
Gibco BRL, Australia
AJAX Chemicals, USA
BDH Chemicals, Australia
Sigma Chemicals, USA
Glycerol
HEPES
Hydrochloric acid (HCl)
Recombinant human interleukin-5 (rhIL-5)
Isoton
Lithium coenzyme A (Li CoA)
Luciferin, sodium salt
Luria-Bertani agar
Magnesium chloride (MgCl₂)
Magnesium sulphate (MgSO₄)
Mini plasmid preparation buffers
10x Molecular weight loading buffer
Molecular weight marker
Monothioglycerol (MTG)
Non-essential amino acids
NP-40
Oligo (dT)₁₅ primers
Penicillin
Phenol:Chloroform:Isoamylalcohol
(25:24:1)
Phorbol-12-myristate-13-acetate (PMA)
Plasmid preparation kit
poly dIdC
Polyethylene glycol (PEG)
Potassium chloride (KCl)
Potassium dihydrogen phosphate
(KH₂PO₄)
Propan-2-ol
Protease inhibitor cocktail
QIAquick Gel Extraction kit
RNAsin
RNeasy Extraction Mini kit
RPMI 1640 medium

ICN Biomedicals Inc., USA
Sigma Chemicals, USA
Univar, APS, Australia
Division of Cell Biology, ICHR, Australia
Coulter Electronics Pty. Ltd., Australia
Sigma Chemicals, USA
Molecular Probes, USA
Sigma Chemicals, USA
AJAX Chemicals, USA
AJAX Chemicals, USA
Bresatec, Australia
Progen Industries Ltd., Australia
Progen Industries Ltd., Australia
Sigma Chemicals, USA
Gibco BRL, Australia
Fluka Biochemika, Switzerland
Promega Corp., USA
Gibco BRL, Australia
Sigma Chemicals, USA
Sigma Chemicals, USA
QIAGEN Inc., USA
ICN Biomedicals Inc., USA
Alltech Associates Pty. Ltd., Australia
May & Baker, Australia
BDH Chemicals, Australia
Merck Pty. Ltd., Australia
Boehringer Mannheim, Germany
QIAGEN Inc., USA
Promega Corp., USA
QIAGEN Inc., USA
Gibco BRL, Australia
Salmon sperm DNA
Skim-milk powder
Sodium acetate (NaCH$_3$COOH)
Sodium chloride (NaCl)
Sodium citrate
Sodium dodecyl sulphate (SDS)
Sodium hydrogen orthophosphate (Na$_2$HPO$_4$)
Sodium hydroxide (NaOH)
Sodium pyruvate
Sodium vanadate (Na$_3$VO$_4$)
Streptomycin
Tris Base
Triton X-100
Tryptone
Xylene cyanol
Yeast extract

2.1.2 Enzymes
Restriction enzymes were purchased from Promega Corporation, USA. Other sources of enzymes include: Shrimp Alkaline Phosphatase from United States Biochemical Corporation (USB); AMV Reverse Transcriptase, T4 Ligase, T4 Polynucleotide Kinase, Tth Polymerase and Klenow Polymerase from Promega Corporation, USA.

2.2 Solutions and Media

2.2.1 Solutions

**Phosphate Buffered Saline (pH 7.4):**
- 2.68mM KCl
- 10.1mM Na$_2$HPO$_4$
- 137mM NaCl
- 1.76mM KH$_2$PO$_4$
TAE buffer: 40mM Tris Acetate
1mM EDTA

TBE buffer: 89mM Tris Base
2mM EDTA
89mM Boric acid

TE buffer (pH 7.5): 10mM Tris Cl (pH 7.5)
1mM EDTA

Annealing buffer: (Stored at −20°C) 10mM MgCl₂
10mM Tris (pH 8.3)

Solutions were made up in sterile glass bottles using ddH₂O then warmed to dissolve any remaining solids. Stock solutions were stored at room temperature unless otherwise specified, and dilutions were made in ddH₂O.

Luciferase buffer: 50mM Tris HCl (pH 7.8)
15mM MgSO₄
33.3mM DTT
0.1mM EDTA (pH 8.2)
0.25mM Li Coenzyme A
0.5mM Na Luciferin
0.5mM Li ATP
0.5% (v/v) Triton X-100

Buffer was made in ddH₂O on ice and stored at −20°C in silver foil-covered vials.

Electrophoresis loading dye: 45% (v/v) Glycerol
55% (v/v) TE buffer
0.25% (w/v) Xylene Cyanol
0.25% (w/v) Bromophenol Blue
Solutions listed below were used in ‘Colony Hybridisation’ (Section 2.16).

**Hybridisation buffer:**
- 50% (v/v) deionised Formamide
- 1% (w/v) Polyethylene Glycol
- 7% (w/v) SDS
- 0.5mg/ml Salmon Sperm DNA
- 0.5% (w/v) Skim-milk powder

Buffer was prepared at 50°C and pre-heated for 60 minutes prior to hybridisation.

**Saline Sodium Citrate buffer:**
- 1.5M NaCl
- 0.15M Sodium Citrate

**Denaturing solution:**
- 500mM NaOH
- 500mM NaCl

**Neutralising solution:**
- 1M Tris HCl (pH 7.5)
- 1.5M NaCl

Solutions listed below were used in ‘Nuclear Extraction’ (Section 2.13) and ‘EMSA’ (Section 2.15).

**200mM Na$_3$VO$_4$:**
1.89g of Na$_3$VO$_4$ were dissolved in 50ml dH$_2$O and the pH adjusted to 10 (solution turns yellow). The solution was boiled until colourless then allowed to cool before readjusting the pH to 10. After a second boiling, and the pH confirmed to be stable at 10, the solution was stored at 4°C. The solution was remade every two weeks.

**Solution A:**
- 10mM HEPES (pH 7.9)
- 10mM KCl
- 0.1mM EDTA
- 0.1mM EGTA
Solution C:
- 20mM HEPES (pH 7.9)
- 420mM NaCl
- 1mM EDTA
- 1mM EGTA
- 20% (v/v) Glycerol

Solution D:
- 20mM HEPES (pH 7.9)
- 0.1M KCl
- 0.2mM EDTA
- 20% (v/v) Glycerol

Solutions A, C and D were made up in ddH₂O in sterile glass bottles and stored at 4°C. The following inhibitors were added to each solution (subsequently called Buffers A, C and D) prior to use for nuclear extractions:
- 0.5mM DTT
- 1mM Na₃VO₄
- 1x protease inhibitor cocktail

2.2.2 Media

2.2.2.1 Bacterial Media

SOC medium:
- 2% (w/v) Tryptone
- 0.5% (w/v) Yeast extract
- 10mM NaCl
- 2.5mM KCl
- 10mM MgCl₂
- 10mM MgSO₄
- 20mM Glucose

Luria-Bertani broth (LB broth):
- 1% (w/v) Tryptone
- 0.5% (w/v) Yeast extract
- 1% (w/v) NaCl
**Luria-Beritani agar (LB agar):**

| LB broth | 1.5% (w/v) Agar Bacteriological |

All ingredients were added to ddH₂O, stirred until dissolved, and autoclaved.

**Ampicillin broth:**

| LB broth | 100µg/ml Ampicillin |

**Ampicillin agar:**

| 3.2% LB agar in ddH₂O | 100µg/ml Ampicillin |

LB agar was completely dissolved and cooled to approximately 60°C before ampicillin was added. Plates were poured and allowed to dry completely before use or storage at 4°C. Plates were made fresh every 2-4 weeks due to deterioration of ampicillin.

### 2.2.2.2 Tissue Culture Media

**Standard media:**

| 500mL RPMI 1640 | 7.5% (v/v) FCS (HI) |
| 2mM L-glutamine | 75µM Monothioglycerol |
| 10mM HEPES | 100µM Non-Essential Amino Acids |
| 1mM Sodium Pyruvate | 100U/ml Penicillin |
| 100µg/ml Streptomycin |

**IL-5 supplemented:**

Same recipe as standard media except only 5% (v/v) FCS (HI) was used, and for the addition of 10-15ng/ml recombinant human IL-5. This media is used to culture BAF-Luciferase cells.
Non-sterile ingredients were filter-sterilized through a 0.22\,\mu m filter unit (Corning, USA) and stored at 4°C until use. Only heat-inactivated (HI) FCS was used, and this was achieved by incubating FCS in 56°C for 30 minutes.

2.3 Plasmid DNA Preparations

2.3.1 Large Plasmid Preparation

Large amounts of plasmid DNA were prepared using the QIAGEN Plasmid Purification System (QIAGEN Inc., USA). DNA quantities of 100, 500 and 2500\,\mu g could be recovered using this system. In short, it involved the use of a series of buffers for alkaline lysis of bacterial cells, removal of chromosomal DNA and cell debris via centrifugation and the running of plasmid containing supernatant through a QIAGEN resin column. The column was washed and the DNA eluted and precipitated. DNA from this system was commonly used for electroporation of PER117 and Jurkat cells as well as preparation of vector DNA. The detailed protocol is outlined in the QIAGEN plasmid handbook.

2.3.2 Mini Plasmid Preparation

5ml of Ampicillin broth was inoculated with a single transformed *E. coli* colony and grown, shaking at 37°C overnight. A portion of this bacterial culture (1.5ml) was spun for 30 seconds at 13,000rpm in a bench microfuge (IEC/Micromax, Selby Scientific and Medical, Australia). The supernatant was discarded and the pellet was resuspended in 100\,\mu l of BPR resuspension buffer (GeneWorks, Australia) and 1\,\mu l RNase A. 100\,\mu l BPL lysis buffer (GeneWorks, Australia) was then added, mixed and incubated at room temperature until the solution is clear. It was then treated with 100\,\mu l BPN neutralization buffer (GeneWorks, Australia), mixed gently and spun for 30 minutes at 13,000rpm. The supernatant was collected and transferred to a second Eppendorf tube (InterPath, Australia). Three volumes of 100% ethanol and 10% volume of sodium acetate were added, the mixture was incubated at -20°C for 10-15 minutes and centrifuged for 15 minutes at 13,000rpm. The supernatant was discarded, the pellet was washed with 70% ethanol, and the mixture was spun for 10
minutes at 13,000rpm. The supernatant was discarded, the pellet was dried and resuspended in 50μl distilled H2O.

2.4 General DNA Manipulations

2.4.1 Ethanol Precipitation
DNA solutions were mixed with 0.1 volumes of 3M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol. The samples were then mixed by inversion and the DNA precipitated at -20°C for approximately 30 minutes. DNA was then recovered by centrifugation at 13000rpm for 15 minutes at 4°C. The pellet was washed with 70%(v/v) ethanol, dried and resuspended in an appropriate volume of ddH2O or TE buffer.

2.4.2 Phenol/Chloroform Extraction
DNA samples were purified by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) as described previously (Sambrook, Fritsch & Maniatis, 1989). This procedure was repeated until no residue was seen, commonly 2-3 times, and then the DNA recovered by ethanol precipitation (Section 2.7.1).

2.4.3 Restriction Enzyme Digestion
DNA samples were digested in solutions of final volumes of 20, 50, or 100μl. The solutions contained 1U restriction enzyme(s) for every 10μg DNA, 0.1 volume of the corresponding 10x restriction buffer, and made up with ddH2O. Samples were then incubated for 60-120 minutes depending on the amount of DNA to be digested, at the optimum temperature recommended for the enzyme(s). Enzymes were heat-inactivated following digestion by incubating at 65°C for 10-15 minutes.

2.4.4 Agarose Gel Electrophoresis
Gel electrophoreses were done in mini-sub, wide mini-sub or sub gel electrophoresis tanks (Bio-Rad Laboratories, USA). The gels (ranging from 1-2% w/v) were prepared from genetic technology grade agarose in 1x TAE buffer. Electrophoreses
were then carried out at 70-90V using either 1kb or 100bp molecular weight ladder as molecular weight marker.

2.4.5 Gel Photography
Following electrophoresis, DNA was visualized on an ultraviolet transilluminator surface (Bi-O-Vision TVD-1000, Medos, Australia). Photographs were taken using a digital camera (Canon PowerShot G1), and processed using Micrografix Picture Publisher or Adobe Photoshop software.

2.5 Preparation of Recombinant Plasmids

2.5.1 Preparation of Vector DNA
DNA was prepared from LB (+Amp) broth cultures inoculated previously from glycerol stock subcultures and plasmid purified as described in Section 2.6.1. The vector DNA was then digested with the appropriate restriction enzyme(s) (Section 2.7.3), run on agarose gel (Section 2.7.4) to remove any undigested DNA and purified using QIAquick gel extraction kit (Section 2.8.5). Following digestion with a single enzyme, vectors were then dephosphorylated (Section 2.8.4) to prevent re-ligation. A small amount of purified cut vector was finally run on a gel to estimate its concentration prior to use in ligation reaction(s).

2.5.2 Preparation of Insert DNA
Plasmid DNA, containing fragment(s) of interest, was prepared in the same manner as that described in Section 2.8.1. The plasmid was digested with the appropriate restriction enzyme(s) (Section 2.7.3) to release the fragment of interest. Digested DNA was run on an agarose gel (Section 2.7.4) to separate the fragment from the cut plasmid and purified from the agarose gel (Section 2.8.5). The concentration of insert DNA was determined and used directly in ligation reaction(s).
2.5.3 Blunt-ending of Plasmid Overhangs

Following digestion of plasmid DNA, it was sometimes necessary to blunt overhanging ends generated by plasmid digestion for subsequent DNA manipulations.

The following were added to purified DNA: 5μl 1x Klenow buffer
2μl dNTPs
2μl Klenow polymerase (5U/μl)

The solution was then made up to 50μl with ddH₂O, incubated at room temperature for 10 minutes followed by heat-inactivation at 75°C for 10 minutes.

2.5.4 Dephosphorylation of Vector DNA

Digested vectors were treated with Shrimp Alkaline Phosphatase to prevent re-circularisation during ligation reactions. This was carried out according to the protocol outlined previously (Sambrook, Fritsch & Maniatis, 1989) with the modification that enzyme inactivation was carried out by incubating digested solutions at 65°C for 15 minutes.

2.5.5 QIAGEN Filter Purification of DNA

Plasmid DNA and DNA fragments run on agarose gels and required from further work was purified by a QIAquick gel extraction kit (QiaGen Inc., USA). The procedure was carried out according to the protocol supplied with the kit. Following elution of DNA with 10mM Tris-HCl (pH 8.5), samples were ethanol precipitated (Section 2.7.1) and re-dissolved in a suitable volume of ddH₂O or TE buffer.

2.5.6 Ligation Reactions

Vector and insert DNA were ligated at molar ratios dependent on the type of ligation. Dephosphorylated vector and insert with compatible ends were ligated at 1:1 (vector:insert) molar ratios. Blunt-ended, dephosphorylated vector and insert were ligated at molar ratios of 1:2 or 1:3 (vector:insert). To these DNA mixtures, 0.5-1μl of T4 DNA Ligase and 2μl of 10x ligation buffer were added and made up to final volumes of 20μl with ddH₂O. Ligation was carried out at 15°C overnight.
Following ligation reactions, the mixtures were used directly in the transformation of chemically competent *E. coli* bacteria (Section 2.9).

### 2.6 Vectors

#### 2.6.1 pSI

The pSI vector by Promega, USA, is an expression vector which has a multiple cloning site incorporating a large number of restriction sites, allowing the vector to be used in sub-cloning procedures. The vectors also contain an SV40 enhancer and early promoter sequences.

#### 2.6.2 pSP72

The pSP72 vector by Promega, USA, is an expression vector which contains a large number of restriction sites in the multiple cloning site flanked by the SP6 and T7 promoters in opposite orientations. The large number of restriction sites makes it an ideal vector for sub-cloning procedures.

#### 2.6.3 pGL3-Basic

The pGL3 series of vectors by Promega, USA, consist of 4 luciferase reporter vectors that provide a basis for the analysis of factors that regulate the expression of mammalian genes. The pGL3-Basic vector contains a multiple cloning site upstream of the Luciferase (Luc) reporter gene which precedes an SV40 poly(A) signal. This vector also lacks eukaryotic promoter and enhancer sequences.

### 2.7 Transformation of Competent Bacterial Strains

#### 2.7.1 Preparation of Competent Bacterial Strain

Chemically competent bacteria were prepared according to the method described previously (Nishimura et al., 1990) using polyethylene glycol (PEG) and magnesium sulphate (MgSO₄).
2.7.2 Transformation of Competent Bacterial Strains
Competent bacteria were taken from −70°C and placed on ice until thawed. The 20μl ligation mixture was then added, mixed and incubated on ice for 40 minutes. The bacteria were then heat shocked for 90 seconds at 42°C before placing immediately on ice for a further 2 minutes. This mixture was then added to 1ml of LB broth which was grown for 1 hour at 37°C. Volumes of 200μl and 800μl of this culture were plated on Ampicillin agar and incubated overnight at 37°C.

2.8 Escherichia coli Host Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>recA1 endoA1 gyrA96 thi hsdR17 supE44 relA1 (lac, pro) F'traD36proABlacF lacZ M15</td>
</tr>
<tr>
<td>DH5-α</td>
<td>F' Phi80dlacZ DeltaM15 Delta(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK-mK+)phoA supE44 lambda- thi-1</td>
</tr>
</tbody>
</table>

2.9 DNA Sequencing
Up to 0.25μg of DNA to be sequenced was added to a 10μl sequencing reaction containing 4μl Terminator Ready Reaction mix (RPH Sequencing Department, Perth), 3.2pmol sequencing primer(s), and dH2O. The reaction was then carried out in a PTC-100 PCR machine (MJ Research Inc., Australia) for 25 cycles, whereby each cycle consisted of heating to 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Samples were then chilled to 4°C before being subjected to ethanol precipitation (Section 2.7.1). Sequencing was performed at the Royal Perth Hospital Immunology Department Sequencing Facility.
2.10 Eukaryotic Cell Culture and Procedures

2.10.1 Cell Culture

PER117 and Jurkat cells were cultured in standard media, and BAF-Luciferase cells were cultured in IL-5 supplemented media. All cells were grown in a humidified atmosphere at 37°C with 5% (v/v) CO₂ in 25cm², 75cm² or 125cm² tissue culture flasks (Nunc, USA).

2.10.2 Transient Transfection of Plasmids

PER117 and Jurkat cells were transfected using a Gene Pulser (Bio-Rad Laboratories, USA). Approximately 10µg of DNA of interest or 10µg of β-galactosidase DNA was added to 10⁷ cells suspended in 400µl standard culture medium in a 0.4cm electroporation cuvette (Bio-Rad Laboratories, USA). β-galactosidase DNA was used as the control used to measure and normalise transfection efficiency between cells, IL-5 promoter-luciferase constructs and treatments. A 10-minute incubation at room temperature then ensued to allow DNA to adhere to the cell walls.

Cells were electroporated at 280V (PER117) and 300V (Jurkat), 960µFd. Following electroporation, cells were incubated for 10-20 minutes at room temperature to allow for recovery. Cells were then dispensed into 5ml standard culture media in 50ml-tissue culture flasks, and were incubated at 37°C in 5% (v/v) CO₂ for 4-6 hours before stimulation.

2.10.3 Cell Stimulation

T-cell activation of PER117 and Jurkat cells was achieved by addition of 10ng/mL phorbol-12-myristate-13-acetate (PMA) alone or in combination with 1mM dibuteryl-cyclic-adenosine-monophosphate (cAMP), 0.25µM calcium ionophore A23187 (Cal), or 0.2µg/mL α-Leu-CD28. In experiments where 50nM Dexamethasone was used as an inhibitor, a 10-minute pre-incubation with the compound preceded cell stimulation in each experiment. Following stimulation,
cells were incubated for 16 hours in 5% (v/v) CO₂ at 37°C before subjected to assays.

2.10.4 Luciferase Assay

The luciferase assay for light production was modified from the commercial protocol described by Promega, USA. The protocol used in these experiments involved no separate lysis step in the assay system. Triton X-100 and Tris HCl were added to the luciferase buffer (See Section 2.2.1), and additionally, tricine and (MgCO₃)₄Mg(OH)₂.5H₂O were omitted from the luciferase buffer. This resulted in a buffer that lysed the cells, thus providing the environment and substrate for the light reaction to ensue in the well of the luminometer assay plate (Wallac, Finland).

Cells to be assayed for luciferase activity were centrifuged and resuspended in 100μl luciferase buffer then added to wells in a black 96-well luminometer plate (Wallac, Finland) and immediately read by Victor 1420 Multilabel counter (Software and counter by Wallac, Finland). Results were read in counts per second (cps).

2.10.5 IL-5 Bioassay

Cells to be tested for IL-5 production were stimulated to produce this cytokine. Following incubation, cultures were spun and supernatants collected for assay. BAF-Luciferase cells were grown in IL-5 supplemented media to the exponential growth phase then washed in standard commercial media and resuspended at 2x10⁵ cells/ml in the same media. In a sterile 96-well clear bottom luminometer plate (Wallac, Finland), doubling dilutions of recombinant human IL-5 in standard commercial media were made across one row (50μl volumes). Doubling dilutions on test supernatants were made across subsequent rows and then 50μl of BAF-Luciferase cells were added to all wells. The plate was then incubated for 48 hours at 37°C in a humidified atmosphere with 5% CO₂.

After incubation, 50μl of luciferase buffer was added to all wells and the plate read in the Victor 1420 Multilabel counter (Wallac, Finland).
2.10.6 Statistical Analysis
Results presented in this thesis have been normalised and are representatives of 3 independent experiments, with error bars representing standard deviations of duplicate transfections at each stimulation point. Means and standard deviations were determined using Microsoft Excel (Microsoft Corporation, USA).

2.11 PCR

2.11.1 Reverse Transcription
PER117 RNA was extracted using RNeasy Extraction kit supplied by Promega. 2.5μg RNA, together with 0.5μg oligo(dT)$_{15}$ primer and dH$_2$O, was incubated at 75°C for 5 minutes. The reaction was immediately put on ice for another 5 minutes, followed by the addition of 2U AMV Reverse Transcriptase enzyme, 5x AMV Reverse Transcriptase buffer, 25mM MgCl$_2$, 10mM dNTPs, and 16U RNASin. The final solution was then incubated at 42°C for 1 hour, and at 95°C for 10 minutes to stop the reaction.

2.11.2 Amplification Method
1μL cDNA was amplified in a solution with a final volume of 100μl. The solution contained 100μmol of each forward and reverse primer (Gibco BRL, Australia), 1mM dNTPs, 10x Tth polymerase buffer, and 1U Tth polymerase enzyme. Distilled water was then added to make the solution up to 100μl.

2.11.2.1 Minimal IL-4 Promoter Fragment Amplification
Samples were amplified by 15 cycles of PCR in a PTC-100 PCR machine (MJ Research Inc., Australia). Each PCR cycle consisted of the linearisation of samples for 15 seconds at 94°C, and annealing for 10 minutes at 68°C. Lastly, the samples were extended further at 72°C for 10 minutes, and cooled down to 4°C (or stored in -20°C) before usage.
2.11.2.2 JunD and Fra2 Fragment Amplification
Samples were amplified by 40 cycles of PCR in a PTC-100 PCR machine (MJ Research Inc., Australia). Each PCR cycle consisted of the linearisation of samples for 1 minute at 94°C, annealing for 1 minute at 66°C, and elongation for 1 minute at 72°C. Lastly, the samples were extended further at 72°C for 10 minutes, and cooled down to 4°C (or stored in -20°C) before usage.

2.11.3 PCR Primers
2.11.3.1 Minimal IL-4 Promoter Primers
Forward: 5' - GGCTCGAGGTGACTGACAATCTGGTG - 3'
Reverse: 5' - CCGGATCCGGTGTCGATTTCGACTTGAC - 3'

2.11.3.2 JunD Fragment Primers
Forward: 5' - CGCACGCTCAACCCCTGCCTTTCC - 3'
Reverse: 5' - CAAACAGGAATGTGGACTCGTACG - 3'

2.11.3.3 Fra2 Fragment Primers
Forward: 5' - GCTTCTACGGTGAGGAGCCCTGCAC - 3'
Reverse: 5' - GGGTTACAGAGCCAGCAGAGTGGCGG - 3'

2.12 Nuclear Extract Preparations

Cells to be extracted (10^7) were collected by centrifugation at 1100rpm for 5 minutes then washed once with 10ml cold PBS and resuspended in 1ml PBS. All subsequent manipulations were carried out on ice. PBS was drained off completely and the pellet resuspended in 600μl cold buffer A (Section 2.2.1), then incubated on ice for 30 minutes. Thirty-eight microliters of 10% NP-40 was added and the mixture vortexed for 10 seconds. The homogenate was centrifuged for 5 minutes at 4500rpm and at 4°C, the supernatant discarded and the pellet resuspended in 80μl cold buffer C (Section 2.2.1) and 2.7μl 5M NaCl. The pellet was rocked vigorously for 30 minutes at 4°C then centrifuged for 5 minutes at 13000rpm and the supernatant snap frozen in 10-20μl aliquots. Aliquots were stored at -70°C until use.
Nuclear extract protein concentration was determined by a Biuret assay from Bio-Rad Laboratories, USA. The assay was carried out according to the manufacturers instructions and optical density recorded on the Victor 1420 Multilabel counter (Wallac, Finland). Standard curves were produced using known BSA dilutions.

2.13 Radiolabelling of Probes

Sense and antisense oligonucleotides were designed and ordered from Gibco BRL (Australia) or Sigma Genosys (Australia), and arrived RPC purified and lyophilised. Oligonucleotides were resuspended in ddH₂O and stored at −20°C. Ten pico moles of sense oligonucleotide was labelled with 50μCi γ-32P-ATP using T4 polynucleotide kinase at 37°C for 30 minutes. The reaction was then n-butanol extracted and the pellet redissolved in ddH₂O. Approximately 2.5pmoles of radiolabelled sense oligonucleotide was then annealed to 5pmoles of antisense oligonucleotide in 1x annealing buffer by heating to 80°C for 10 minutes then cooling slowly to room temperature. The double stranded radiolabelled oligonucleotide was then diluted 1/10 in 1x annealing buffer (12.5fmol/μl) and stored at −20°C.

2.14 Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic Mobility Shift Assays (EMSA) were carried out at 4°C using vertical slab gel units (Hoefer Scientific Instruments, USA). 6% polyacrylamide gels in 1x TBE buffer were prepared and run in 0.5x TBE buffer.

Three micrograms of nuclear extract protein were incubated for 30 minutes on ice with 25fmol radiolabelled-oligonucleotide, 1mM DTT, 7.5mM MgCl₂ and 0.5μg/μl poly dIdC in buffer D (Section 2.2.1). The entire reaction volumes were loaded onto the polyacrylamide gel and run at 180V for 3 hours.
Gels were dried in a Model 583 Gel Dryer (Bio-Rad Laboratories, USA) for 1 hour and transferred to EC-AWU cassettes (Fuji, Japan) with diagnostic film (Eastman Kodak Company, New York) and exposed for 16 hours at −70°C. EMSA images were generated using a Canon PowerShot G1 Digital Camera, and processed using Micrografx Picture Publisher or Adobe Photoshop software.

2.15 Colony Hybridisation

2.15.1 Preparation of Nitrocellulose Membranes

Bacterial colonies that contained the desired mutant were identified through screening by a hybridisation method described previously (Grunstein & Hogness, 1975). A nitrocellulose membrane (Amersham International, England), pre-wet on a fresh LB/Amp plate, was placed over the colonies (grown overnight at 37°C) and the membrane was marked asymmetrically with the underlying medium. The membrane was then peeled from the plate, placed colony side up on a fresh plate, and the colonies grown at 37°C for 1 to 3 hours until approximately 2-3mm in size. The bacterial colonies were then lysed and the liberated DNA bound to the membrane by placing it, colony side up, on 3MM Whatmann paper (Whatmann, England) saturated with denaturing solution for 20 minutes. Following transfer to 3MM Whatmann paper saturated with neutralising solution for 12 minutes, the filter was gently blotted, colony side down, on 3MM Whatmann paper to remove excess colony debris. The membrane was then air dried and placed on a UV chamber (GS Gene Linker, Bio-Rad Laboratories, USA) for 10 minutes.

2.15.2 Hybridisation

The membranes were pre-hybridised on 'Rapid-Buffer' (Amersham International, England) for one hour at 65°C. The radiolabelled probe (Section 2.14) was then added to a Hybaid hybridisation bottle (Hybaid, England) (to a concentration of 10ng/ml) and the hybridisation carried out at 65°C for 1 hour.
2.15.3 Washing of Nitrocellulose Membranes

Following hybridisation, the membrane was rinsed briefly in 2x SSC buffer. The membrane was then washed for 20 minutes in 2x SSC+0.1% (w/v) SDS at room temperature. Then it was washed 2x15 minutes in 1x SSC+1% (w/v) SDS and finally transferred to a 0.2x SSC+1% (w/v) SDS solution where it was incubated at 42°C for 15 minutes. Following washing, the membrane was blotted dry with 3MM Whatmann paper and wrapped in Glad Wrap. Exposure of the membrane to Kodak XK-1 film was carried out at -70°C for ~24-48 hours.
CHAPTER 3

IL-5 CLE0 ACTIVITY
3.1 Experimental Approach

This study has compared the relative activities of a reporter gene construct driven by the IL-5 GATA/CLE0/TATA (G/C/T) elements and the IL-5 CLE0/TATA (C/T) elements in an effort to clarify the role of the CLE0 element in hIL-5 gene transcription.

The G/C/T-IL-5 reporter construct (Fig. 3.1A) was generated using an oligonucleotide (~80 to +44) with a single base change at position -60 that introduced a Csp45I restriction site, and with modified ends to introduce NheI and NcoI sites for cloning into the NheI/NcoI site of pGL3-Basic vector. The oligonucleotide was then ligated into the pGL3-Basic vector in which the Csp45I enzyme site was disrupted by a single base mutation at position 257, using the Altered Sites® II In Vitro Mutagenesis System (Promega, USA).

The G/C/T-IL-5 construct was further digested with Csp45I and NheI to remove the GATA element, blunt-ended, and re-ligated to generate the C/T-IL-5 promoter construct (~60 to +44; Fig. 3.1B). The activities of the G/C/T- and C/T-IL-5 constructs were then observed following transient transfection in both the PER117 and Jurkat cell lines (Section 1.5.2.2).

Previous deletion studies have shown that a variety of other factors and elements that modulate hIL-5 expression are also found between -509 to +44 respective of the transcription initiation site (Section 1.2.3). This laboratory has previously created the 509bp-IL-5 reporter construct (Fig. 3.1C; Schwenger, G, unpublished data) where a fragment from position -509 to +44 of the hIL-5 promoter was ligated into NheI/BglII-digested pGL3-Basic. This construct was used as a positive control in the transient transfection experiments.
**Figure 3.1:** The A) G/C/T-IL-5 and B) C/T-IL-5 promoter constructs, created using pGL3-Basic vector as backbone vector. The C) 509bp-IL-5 promoter construct was previously created by Dr. G. Schwenger (unpublished data). Constructs are not drawn to scale. D) Schematic representation of the \(-89\)bp-IL-5 fragment used by Dr. M. DeBoer (unpublished data) in transient transfection of PBMC.
3.2 Results

3.2.1 T-cell Lines

Transient transfection of the G/C/T-IL-5, C/T-IL-5 and 509bp-IL-5 constructs showed no significant difference of expression in unstimulated PER117 cells (Fig. 3.2A). The addition of PMA into the culture resulted in increased expression from the 509bp-IL-5 construct (Fig. 3.2A). This increase was nearly 100-fold, illustrating the inducibility of IL-5 gene expression. A similar induction was achieved by the C/T-IL-5 construct although the G/C/T-IL-5 construct gave a higher induction compared with the other constructs, suggesting a positive effect of PMA on the GATA element in this construct.

The addition of cAMP doubled expression of the PMA-stimulated C/T-IL-5 construct (Fig. 3.2A), but had little effect on the expression of the G/C/T-IL-5 construct. This suggests that the GATA element is unresponsive to cAMP signalling pathway. In contrast, the cAMP co-stimulation had only a slight effect on the 509bp-IL-5 construct. The responsiveness of the CLE0 element to both PMA and cAMP stimulations suggest an important role of hIL-5 CLE0 as a powerful activator.

Ca²⁺ co-stimulation with PMA, however, had the opposite effect to that of cAMP co-stimulation (Fig. 3.2A). A significant reduction in expression was observed for all three constructs, suggesting that neither the GATA and CLE0 elements are responsive to Ca²⁺ stimulation in PER117 cells.

The addition of αCD28 had no effect on reporter gene expression in any of the constructs compared to PMA-only stimulation (Fig. 3.2A). This suggests that none of the regulatory elements present within the 509bp region, including the GATA and CLE0 elements, were responsive to αCD28-stimulation, and that the CD28-responsive element maybe present outside of this region.
A) PER117 cell line

B) Jurkat cell line

Figure 3.2: Transient activity of minimal hIL-5 promoter in A) PER117 and B) Jurkat cell lines. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
When the G/C/T-IL-5, C/T-IL-5 and 509bp-IL-5 constructs were transfected into Jurkat cells, only low levels of expression were observed compared to those obtained in PER117 cells. This is consistent with the fact that these cells do not express IL-5 (Fig. 3.2B). In each method of stimulation, however, the reporter gene expression from the C/T-IL-5 construct was higher than those of G/C/T-IL-5 and 509bp-IL-5 constructs. Furthermore, there was a relatively strong induction of luciferase activity from the C/T-IL-5 construct following PMA/cAMP stimulation. Although the reason for this difference in expression was not clear, the result emphasizes that hIL-5 CLE0 is a powerful activator as it is active in a cell line that does not express IL-5 protein.

3.2.2 Primary T-cells

Previous work in this laboratory (de Boer, M; unpublished data) showed a construct containing hIL-5 proximal promoter region up to position −89 has activity in PHA-stimulated PBMCs. The −89bp-IL-5 construct differed from the G/C/T-IL-5 construct by the inclusion of 9 base pairs in positions −81 to −89 (Fig. 3.1D)), which contain part of the hIL-5 PRE1 repressor element. This construct, however, generated a higher reporter expression than both the 509bp-IL-5 construct (Fig. 3.3) and a longer construct containing a complete PRE1 element (de Boer M; unpublished data). By disrupting the PRE1 element, the −89bp-IL-5 construct showed that the hIL-5 CLE0 and GATA elements function in primary T-cells.

3.3 Discussion

The 5’-flanking region of the IL-5 RNA initiation site contains the TATA-box with the GATA and CLE0 regulatory elements located immediately upstream (Fig. 1.5). Mutations introduced within these elements abolish IL-5 expression, indicating that these elements are essential for promoter activity (Naora et al., 1994; Thomas, Mordvinov & Sanderson, 1999; Gruart-Gouilleux, Engels & Sullivan, 1995; Lee et al., 1998; Zhang et al., 1997). The PRE1, PRE2 and another GATA element at position −400 were found to significantly suppress IL-5 gene transcription (Schwenger et al., 1999; Schwenger & Sanderson, 1998; Schwenger et al., 2001),
Figure 3.3: Transient activity of hIL-5 proximal regulatory elements in PHA-stimulated PBMC. Result was a representative of experiments performed by Dr. M. DeBoer (unpublished data).
and deletion of these elements, leaving only the proximal GATA and CLE0 elements, increased construct activities in PER117 cells (Fig. 3.2) and primary human T-cells (Fig. 3.3). This result indicates the importance of GATA and CLE0 elements in hIL-5 regulation as the presence of these two elements were enough to induce reporter gene expression.

The importance of IL-5 GATA(−70) and CLE0 has been questioned in several studies; it has been reported that these elements are not involved in IL-5 gene regulation (Ogawa et al., 2002; Stranick et al., 1997; Tsuruta et al., 1995). Earlier observations in mouse cells showed that an NF-AT binding site from −123 to −92 appeared necessary for the full expression of the hIL-5 promoter (Prieschl et al., 1995; Stranick et al., 1997). The difference between these observations and the result of this study may be due to the use of human promoter constructs in mouse cell lines.

Another study in mouse EL4 lymphoma cells, however, showed that the region between −117 to −92 of the mIL-5 promoter is indispensable for PMA/cAMP-stimulated promoter activity (Tsuruta et al., 1995). This observation disagreed with previous studies in different mouse cell lines that showed a region extending from −55 to −41 is essential for mIL-5 promoter activity (Naora et al., 1994; Lee et al., 1998). Furthermore, our group showed that mIL-5 promoter activity is strongly affected when mIL-5 CLE0 competing oligonucleotides were added, indicating the mIL-5 CLE0 element is indispensable for mIL-5 gene regulation (Karlen, D’Ercole & Sanderson, 1996).

Recently, an observation that a hIL-5 fragment from −79bp to +4 did not express in primary human T cells, whereas a construct from −119bp to +4 expressed in an inducible fashion, led the authors to propose that the NFAT site at −110bp is the key to hIL-5 expression (Ogawa et al., 2002). This NFAT site has previously been identified as an activator for hIL-5 expression in both primary T cells and PER117 cells, but was not thought to be the crucial ‘on-off’ switch for hIL-5 transcription (de Boer et al., 1999). Furthermore, other studies have also shown that hIL-5 CLE0 is important for the transcription of hIL-5 gene in both human T-cell clones (Mori et al., 1997) and non-transformed human T-cells (Mori et al., 2000b). Thus, it is
unlikely that the difference between the result of this study and those observed by Ogawa and colleagues (Ogawa et al., 2002) occurred from differences in the cells used.

Interestingly, a comparison of expression of G/C/T-IL-5 and C/T-IL-5 gave an unexpected result in that removal of GATA gave slightly higher expression (Fig. 3.2A). Taken on their own these data would suggest that this GATA element has little if any activity. However, mutation of the element in the context of the 509bp promoter reduced expression to very low levels (Schwenger et al., 2001). Furthermore, co-stimulation with αCD28 had no additional effect on any construct expressions (Fig. 3.2A), suggesting that none of the regulatory elements present within the 509bp region were responsive to αCD28-stimulation. Nevertheless, αCD28 has been shown to activate the GATA-3 factor in mice and rats (Rodriguez-Palmero et al., 1999; Heath et al., 2000; Grogan et al., 2001). Thus, a more likely interpretation is that the GATA(−70) element is not active when removed from the context of the full promoter. The more general role of GATA in chromatin structure has been well documented in the mouse (Ferber et al., 1999; Lee et al., 2000; Miyatake, Arai & Arai, 2000; Takemoto et al., 2000), and may well be conserved in the human locus, although the Th1/Th2 distinction is less clear in human T cells.

The study further shows that the CLE0/TATA elements give strong inducible expressions in stimulated PER117 cells (Fig. 3.2A), particularly under PMA/cAMP stimulation. IL-5 CLE0 contains the binding sequences for AP1, and cell stimulation using PMA and a co-stimulatory compound lead to the formation of an AP-1 complex consisting of JunD and Fra-2 proteins in PER117 cell line (Thomas, Mordvinov & Sanderson, 1999). In addition, our group observed that the mRNA (Kok CC, unpublished data) and protein levels of Fra2 transcription factor (Schwenger et al., 2002) increased with PMA stimulation, and were enhanced by addition of cAMP. When a similar observation was noted in the Jurkat cell line, a cell line that does not produce IL-5 protein (Fig. 3.2B), the results indicate that IL-5 CLE0 is a powerful activator that functions through the cAMP co-stimulatory pathway.
The above findings concur with the results of others whose experiments subjected both human and mouse cell lines to the same co-stimulatory compounds. In SP-B21 human T-cell clones (Watanabe et al., 1994) and EL4 mouse lymphoma cells (Gruart-Gouilleux, Engels & Sullivan, 1995; Karlen, D'Ercole & Sanderson, 1996; Lee et al., 1993), it was shown that the activation of the IL-5 gene can be triggered by cAMP acting synergistically with PMA.

The high conservation of IL-5 proximal promoter elements GATA and CLE0 between species, compared with the low conservation of other elements within the −509/+44 IL-5 promoter region, suggests an important role for GATA and CLE0 (Fig. 1.4). The present results show that although GATA may be more active in the context of a full hIL-5 promoter, the hIL-5 CLE0 alone is a remarkable activator. To further clarify the role(s) of hIL-5 CLE0, its ability to activate a heterologous promoter will be determined in the next chapter.
CHAPTER 4

EFFECT OF IL-5 CLE0 ON A HETEROLOGOUS PROMOTER
4.1 Experimental Approach

In this chapter, the roles of IL-5 CLE0 are further clarified; firstly, its ability to transactivate a heterologous promoter was investigated. Secondly, as individual promoter elements can often impart enhancer activity if multiple copies of that element are inserted at a more distant location (Carey & Smale, 2000), this study analysed the ability of IL-5 CLE0 to act as an enhancer in both forward- and reverse-orientations. The conservation of the proximal region of the IL-5 promoter between different species (Fig. 1.4) also led to the analysis of the enhancing and repressive abilities of the GATA(−70) and PRE1 elements, respectively.

The proximal region of the human IL-4 promoter was selected as a heterologous promoter as it had basal promoter activity (Section 1.4.1). To create the heterologous promoter, a 160bp hIL-4 promoter DNA fragment spanning from position −100 to +60 relative to transcription initiation site was amplified by PCR. The amplified fragment was sub-cloned into pSP72 vector before being ligated into BglII/HindIII sites of pGL3-Basic vector, producing the 160bp-IL-4 promoter construct (Fig. 4.1A).

To determine whether IL-5 CLE0 can act as an enhancer, oligonucleotides containing copies of forward- (Fig. 4.1B-D) or reverse-oriented (Fig. 4.1G) CLE0 sequence were ligated upstream of the 160bp-IL-4 promoter. The abilities of the GATA(−70) and PRE1 elements were analysed by ligating 3 copies of GATA(−70) or 3 copies of PRE1 sequences upstream of the 160bp-IL-4 promoter construct (Fig. 4.1E-F). The resulting promoter constructs were then transiently transfected into PER117 and Jurkat cells.
A) 160bp-IL-4 promoter construct

B) 1x-IL-5 CLE0/IL-4 promoter construct

C) 2x-IL-5 CLE0/IL-4 promoter construct

D) 3x-IL-5 CLE0/IL-4 promoter construct
Figure 4.1: A) The minimal hIL-4 promoter construct used as heterologous promoter. B) A single copy of forward-oriented hIL-5 CLE0 element, C) 2 copies of forward-oriented hIL-5 CLE0 element, D) 3 copies of forward-oriented hIL-5 CLE0 element, E) 3 copies of hIL-5 GATA(-70) element, F) 3 copies of hIL-5 PRE1 element, and G) 3 copies of reverse-oriented hIL-5 CLE0 element were ligated upstream of the heterologous promoter in pGL3-Basic vector backbone. Constructs are not drawn to scale.
4.2 Results

4.2.1 Heterologous Promoter Activity

The activity of the 160bp-IL-4 promoter construct, which included the IL-4 CLE0-like element, was compared to the activity of the C/T-IL-5 and the 509bp-IL-5 constructs in transient transfection experiments in PER117 and Jurkat cells. As shown previously (Chapter 3), the C/T-IL-5 and 509bp-IL-5 constructs gave high levels of inducible expression in PER117 cells (Fig. 4.2A). In contrast, the 160bp-IL-4 construct gave very low levels of expression under all conditions of stimulations (Fig. 4.2A).

In Jurkat cells, the 160bp-IL-4 construct gave very low levels of expression (Fig. 4.2B), in contrast to higher levels of expression of the C/T-IL-5 construct. Thus, in the two cell lines the 160bp-IL-4 promoter is effectively inactive, and so could be used as a heterologous promoter to study the roles of IL-5 CLE0.

4.2.2 Effect of 1, 2 and 3 Copies of hIL-5 CLE0 on Heterologous Promoter

To study the ability of hIL-5 CLE0 to act as an enhancer, 1, 2 or 3 copies of CLE0 were inserted upstream of the 160bp-IL-4 promoter (Fig. 4.1B-D), and the resulting constructs were transiently transfected into PER117 and Jurkat cells. In unstimulated PER117 cells, the constructs produced little luciferase expression (Fig. 4.3A). Following cell stimulation with PMA, the presence of a single copy of hIL-5 CLE0 doubled the activity of the 160bp-IL-4 promoter (Fig. 4.3A). The insertion of 2 and 3 copies of hIL-5 CLE0 increased the activity of the heterologous promoter by more than 30 and 70-fold, respectively.
Figure 4.2: Transient activity of minimal hIL-4 promoter as a heterologous promoter in A) PER117 and B) Jurkat cell lines. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
A) PER117 cell line

- 160bp-IL-4 promoter
- 2x-IL-5 CLE0/IL-4 promoter
- 1x-IL-5 CLE0/IL-4 promoter
- 3x-IL-5 CLE0/IL-4 promoter

B) Jurkat cell line

- 160bp-IL-4 promoter
- 2x-IL-5 CLE0/IL-4 promoter
- 1x-IL-5 CLE0/IL-4 promoter
- 3x-IL-5 CLE0/IL-4 promoter

Figure 4.3: Transient activity of multimerized hIL-5 CLE0 in A) PER117 and B) Jurkat cell lines. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
The addition of cAMP gave no increase in reporter gene expression (Fig. 4.3A), whereas addition of CaI reduced the activity of all hIL-5 CLE0 constructs, although it did not affect the activity of the heterologous promoter itself (Fig. 4.3A). The latter observation confirms previous results showing that PMA/CaI co-stimulation reduces IL-5 CLE0 activity (Fig. 3.2A). Taken together, these results suggest that the enhancer activity of IL-5 CLE0 is insensitive to the cAMP and Ca^{2+}-signalling pathways.

CD28 co-stimulation also had no additional effect on the activities of the constructs over those obtained through PMA-stimulation alone (Fig. 4.3A). This suggests that neither the hIL-5 CLE0 nor the heterologous promoter is responsive to the CD28-signalling pathway, which agrees with previous observations of the effect of CD28 on the IL-4 (Fig. 4.2A) and the IL-5 promoters (Fig. 3.2A). The presence of a single or more copies of IL-5 CLE0, however, still increased activity above that of the 160bp-IL-4 promoter alone. These results, therefore, support the action of IL-5 CLE0 as an enhancer, though not one that can be activated through the CD28-signalling pathway.

A similar effect of hIL-5 CLE0 was also seen in Jurkat cells, although the levels of expression were much lower (Fig. 4.3B). When the cells were stimulated with PMA, activities of the 160bp-IL-4 and the 1x-IL-5 CLE0/IL-4 constructs were not significantly affected, but the activities of the 2x- and the 3x-IL-5 CLE0/IL-4 constructs were increased by 3 and 9-folds, respectively (Fig. 4.3B). The addition of co-stimulants, however, had no additional effect over PMA-stimulated reporter gene expression. These results show that, although the effects were much smaller in magnitude, hIL-5 CLE0 can act in cells that do not express hIL-5, and confirm that expression is not affected by cell co-stimulation.

Individual promoter elements can convey enhancer activity if multiple copies of that element are inserted at a more distant location (Section 4.1). In this study, IL-5 CLE0 was moved further from its normal position in the IL-5 gene, with respect to the transcription initiation site, upstream of a heterologous. The results of the transient transfections showed that the influence of hIL-5 CLE0 on the heterologous promoter increases with the number of copies present. Therefore, although it can
only be activated through the PMA-signalling pathway, hIL-5 CLE0 can act as an enhancer.

### 4.2.3 Effect of 3 Copies of hIL-5 GATA(−70) and hIL-5 PRE1 on Heterologous Promoter

Immediately upstream of the hIL-5 CLE0 element, two more regulatory elements exist; the GATA(−70) element, a GATA-3 transcription factor binding site at position −70, and the PRE1 element, a repressor element that contains binding sites for YY1, Oct1 and Oct-like transcription factors (Fig. 1.3). As the IL-5 GATA(−70) element was previously shown to be an activator (Schwenger et al., 2001), this study examined the ability of this element to act as an enhancer by transiently transfecting the 3x-IL-5 GATA/IL-4 construct (Fig. 4.1E) into PER117 and Jurkat cell lines. The activity of IL-5 PRE1 element was also examined by using the 3x-IL-5 PRE1/IL-4 construct (Fig. 4.1F) in transient transfection experiments.

#### 4.2.3.1 Effect of 3 Copies of hIL-5 GATA(−70) on Heterologous Promoter

In contrast to the effect of hIL-5 CLE0 (Fig. 4.4A and Section 4.2.2), the 3x-IL-5 GATA/IL-4 construct gave unexpected activities; in unstimulated PER117 cells, the insertion of multiple copies of IL-5 GATA(−70) element nearly halved the basal activity of the 160bp-IL-4 construct (Fig. 4.4B). The addition of PMA into the cell cultures tripled the activity of the 3x-IL-5 GATA/IL-4 construct; yet the level reached was only 30% of that reached by the PMA-stimulated 160bp-IL-4 construct. A similar trend was also observed in PMA/cAMP-stimulated cells, demonstrating the action of cAMP on the heterologous promoter, and not on the IL-5 GATA(−70) element, which agrees with previous observations (Fig. 3.2).

The inhibitory action of IL-5 GATA(−70) was also noted when Cal or α-CD28 was used as a co-stimulant to PMA. The reporter activity of the PMA-stimulated 3x-IL-5 GATA/IL-4 construct were halved with the addition of Cal or α-CD28 co-stimulant (Fig. 4.4B), and they were only 15% and 35%, respectively, of the activity of the 160bp-IL-4 construct under the same stimulations. The results, therefore, suggested a repressive effect of multiple copies of IL-5 GATA(−70).
Figure 4.4: Transient activity of multimerized GATA(-70) and PRE1 elements in PER117 cell line. Representative data from one experiment shown A) with or B) without 3x-IL-5 CLE0/IL-4 promoter construct. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
Figure 4.5: Transient activity of multimerized GATA(-70) and PRE1 elements in Jurkat cell line. Representative data from one experiment shown A) with and B) without 3x-IL-5 CLE0/IL-4 promoter construct. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
The addition of three copies of IL-5 GATA(−70) element also reduced the 160bp-IL-4 promoter activity by 84% in unstimulated Jurkat cells (Fig. 4.5). Cell stimulation had no additional effect on the 3x-IL-5 GATA/IL-4 promoter construct, which exhibited lower activity than the 160bp-IL-4 construct (Fig. 4.5B). Although the effect was small, these results suggest that IL-5 GATA(−70) element can act as a repressor element. This subject will be discussed further in Section 4.3.

4.2.3.2 Effect of 3 Copies of hIL-5 PRE1 on Heterologous Promoter
The insertion of three copies of the IL-5 PRE1 element, a known IL-5 repressor element, upstream of the heterologous promoter, had no additional effect on the activity of the 160bp-IL-4 promoter in unstimulated PER117 cells (Fig. 4.4). The 3x-IL-5 PRE1/IL-4 construct was induced in PMA-stimulated cells, but this activity was 40% lower than the activity of PMA-stimulated 160bp-IL-4 construct (Fig. 4.4B). In the PMA/CaI-stimulated PER117 culture, the addition of the IL-5 PRE1 elements effectively reduced the 160bp-IL-4 promoter activity by 60% (Fig. 4.4B). This suggests that the multimerized IL-5 PRE1 elements were still acting as repressors, and that this repression can occur through both PMA and CaI signalling pathways.

When α-CD28 was added to PMA-stimulated PER117 cells, the reduced activity of the 160bp-IL-4 promoter was further reduced by the multimerized PRE1 elements (Fig. 4.4B). However, this reduction was only 40%, which is comparable to the reduction observed in PMA-stimulated cultures, suggesting that the CD28-signalling pathway exerts its effect principally on the 160bp-IL-4 promoter, and did not have any significant effect on the multimerized PRE1 elements.

Thus far, the activities of the 3x-IL-5 PRE1/IL-4 construct indicated its effectiveness as a negative control in the transient transfection experiments. The three copies of IL-5 PRE1 repressor element inhibited the activities of the 160bp-IL-4 promoter under most cell stimulations except PMA/cAMP. The 3x-IL-5 PRE1/IL-4 construct was observed to be three times more active than the 160bp-IL-4 construct in PMA/cAMP-stimulated PER117 cells. The levels of non-inhibition, however, varied over three repeated experiments, and the reason for the differences in expression could not be explained.
The addition of three copies of IL-5 PRE1 elements also reduced the 160bp-IL-4 promoter activity by 55% in unstimulated Jurkat cells, and cell stimulations did not appear to have any additional effects (Fig. 4.5). These results show that the IL-5 PRE1 can repress the activity of a heterologous promoter.

4.2.4 Effect of Inverted Repeats of hIL-5 CLE0 on Heterologous Promoter

The hIL-5 CLE0 element has been shown to have activation capabilities in a position-independent manner. To determine whether hIL-5 CLE0 can function in an orientation-independent manner, the activity of 3 copies of reverse-oriented hIL-5 CLE0 (3x-IL-5 CLE0-R/IL-4 construct; Fig. 4.1G) was compared to the activity of 3 copies of forward-oriented hIL-5 CLE0 (3x-IL-5 CLE0/IL-4 construct) in PER117 cells.

As shown in Figure 4.6A, the 3x-IL-5 CLE0/IL-4 constructs gave results similar to those observed previously (Fig. 4.3A). On the other hand, the 3x-IL-5 CLE0-R/IL-4 construct had very little effect in comparison to the 160bp-IL-4 construct (Fig. 4.6A) or the 1x-IL-5 CLE0/IL-4 construct (Fig. 4.6B). Thus, while hIL-5 CLE0 is a strong activator, it cannot be regarded as a ‘classical’ enhancer.

4.3 Discussion

The cytokine genes IL-4, IL-5 and IL-13 are closely linked, and the co-expression of these cytokines in TH2 immune responses and in Th2-skewed murine T cells has altered the focus of research towards mechanisms controlling coordinate expression. In human diseases where both IL-4 and IL-5 were present, they were rarely produced by the same T-cells (Mori et al., 2001). Furthermore, eosinophilia can occur without an increase in serum IgE level or expansion of other cell types.
A) 3x-IL-5 CLE0 vs 3x-IL-5 CLE0-R

- 160bp-IL-4 promoter
- 3x-IL-5 CLE0/IL-4 promoter
- 3x-IL-5 CLE0-R/IL-4 promoter

Luciferase fold induction

U/S  | PMA  | PMA/cAMP | PMA/Cal | PMA/CD28

B) 1x-IL-5 CLE0 vs 3x-IL-5 CLE0-R

- 160bp-IL-4 promoter
- 1x-IL-5 CLE0/IL-4 promoter
- 3x-IL-5 CLE0-R/IL-4 promoter

Luciferase fold induction

U/S  | PMA  | PMA/cAMP | PMA/Cal | PMA/CD28

**Figure 4.6:** Transient activity of 3 copies of reverse-oriented multimerized hIL-5 CLE0 in comparison to A) 3 copies of forward-oriented multimerized hIL-5 CLE0, and B) 1 copy of forward-oriented hIL-5 CLE0 in PER117 cell line. Transfections were performed as described in Section 2.10. U/S denotes unstimulated cells.
The above observations, therefore, demonstrated the existence of independent regulatory mechanisms for each gene. For example, the CLE0 elements of different human cytokine genes show varying homology. The highest homology is seen between IL-5 (Mordvinov et al., 1999b) and GM-CSF (Nimer et al., 1996), which differ by only one base, but GM-CSF is not regarded as a Th2 cytokine even though it is found in the Th2 cluster. The IL-4 CLE0 has only low homology with IL-5 (de Boer et al., 1999) and binds different transcription factors (Hodge, Rooney & Glimcher, 1995; Li-Weber, Giasi & Krammer, 1998). Furthermore, the 160bp fragment used in the present study, which contains the CLE0 element, has no transcriptional activity (Abe et al., 1992) (Fig. 4.2).

In contrast, the hIL-5 CLE0 is a strong transcriptional activator in the absence of other promoter elements, as shown by the activity of the C/T-IL-5 construct (Fig. 4.2) and the activation of the hIL-4 minimal promoter (Fig. 4.3). This result concurs with previous observations that multiple copies of hIL-5 CLE0 can activate a thymidine kinase promoter in Jurkat (Yamagata et al., 1997) and non-transformed human T-cells (Mori et al., 2000b).

This study also shows that the hIL-5 CLE0 has little activity in reverse orientation and so cannot be considered a classical enhancer (Fig. 4.6). Furthermore, the enhancing effect of hIL-5 CLE0 was not affected by the addition of co-stimulants, suggesting that IL-5 CLE0 acts only through the PMA-signalling pathway.

The conservation of the proximal region of the IL-5 promoter between different species (Fig. 1.4) led to the analysis of the enhancing and repressive abilities of the GATA(-70) and PRE1 elements, respectively. As the PRE1 element is present in all known IL-5 genes (Fig. 1.4), it is possible that the role of this element is specific to the regulation of the IL-5 gene (Sanderson et al., 1999). However, this study shows that multiple copies of the PRE1 element had only a small repression effect (Figs. 4.4-4.5), suggesting that it may only act as a repressor when it is in its normal position in the hIL-5 gene.

Interestingly, although the GATA(-70) element, adjacent to CLE0, is an important positive regulator in hIL-5 expression (Chapter 3), the multimerized construct did not
increase expression of the heterologous promoter. In fact, it significantly decreased expression (Figs. 4.4-4.5). There are two possible explanations for this; firstly, in mouse cells, the GATA-3 transcription factor was shown to transactivate IL-5 but not IL-4 gene promoters (Ferber et al., 1999; Kishikawa et al., 2001; Lee et al., 2000). In NF-ATc−/−/RAG−/− knockout mice, it was observed that IL-4 gene expression was defective although a full compliment of GATA-3 activity was present (Yoshida et al., 1998). Secondly, in contrast to the GATA(−70) element, another GATA element found at position −400 in the hIL-5 promoter is a powerful repressor (Schwenger et al., 2001). Taken together, these observations suggest that the activity of the GATA(−70) element may be position-dependent.
CHAPTER 5

ANTISENSE TRANSCRIPTION FACTOR EFFECT ON IL-5 CLE0
5.1 Experimental Approach

Protein-DNA binding is important in hIL-5 CLE0 activation as mutations in the sequence abolish hIL-5 expression (Thomas, Mordvinov & Sanderson, 1999; Naora et al., 1994; Gruart-Gouilleux, Engels & Sullivan, 1995). Understanding the rate-limiting transcription factor would give better understanding of the regulation of hIL-5 expression, and possibly identify a potential drug target. Using an antisense approach that has been used previously to successfully block GATA-3 expression (Finotto et al., 2001), constructs were generated to block the expression of the CLE0-binding factors.

It has been shown that Oct1 and Oct2, as well as JunD and Fra2 bind to CLE0 in PER117 cells (Thomas, Mordvinov & Sanderson, 1999). This study investigates the importance of the CLE0-binding transcription factors by reducing their expression in PER117 cells, and observing the effects on the 509bp-IL-5, G/C/T-IL-5 and C/T-IL-5 promoters. Empty pSI construct was used as a negative control since the antisense expression constructs were created using the pSI vector backbone (Promega).

To create antisense Oct1 expression constructs, the complete Oct1 gene was excised from pCG-Oct1 plasmid (Tanaka & Herr, 1990) at the XbaI/BamHI sites, and ligated in reverse orientation into the EcoRI/XbaI sites of pSI vector, producing the FL-ASOct1 expression construct (Fig. 5.1A). A sub-cloning step, however, was required in creating the antisense Oct2 expression construct. Initially, the complete Oct2 gene was ligated in reverse orientation into pSP72 vector (at its XbaI/BamHI sites) from the pCG-Oct2 plasmid (Tanaka & Herr, 1990), generating the pSP-ASOct2 construct. This construct was then digested with AccI and EcoRI enzymes, and the fragment was ligated into the pSI vector, producing the FL-ASOct2 expression construct (Fig. 5.1B).
A) FL-ASOct1 expression construct

B) FL-ASOct2 expression construct

C) SL-ASJunD expression construct

D) SL-ASFra2 expression construct

Figure 5.1: The A) full-length antisense Oct1, B) full-length antisense Oct2, C) short-length antisense Fra2 and D) short-length antisense JunD expression constructs created using pSI vector as backbone vector. The short-length antisense expression constructs were created by Dr. C. C. Kok (unpublished data). Constructs were not drawn to scale.
In creating the antisense JunD and antisense Fra2 constructs, short JunD and Fra2 fragments (292bp and 224bp respectively) were amplified from cDNA obtained from PER117 cells (Kok, CC, unpublished data). The fragments were initially cloned into pGEM-T vectors before finally ligated into the XhoI/SalI sites of pSI vectors (Fig. 5.1C-D). The short antisense JunD and Fra2 constructs are labelled SL-ASJunD and SL-ASFra2 respectively.

5.2 Results

5.2.1 Effect of Full-length Antisense Oct1 and Oct2 on IL-5 CLE0

This section investigates the importance of the Oct1 and Oct2 transcription factors by reducing their expression in PER117 cells, and observing the effects on the 509bp-IL-5, G/C/T-IL-5 and C/T-IL-5 promoters.

5.2.1.1 Effect of Antisense Oct1 on IL-5 CLE0

In stimulated cells (Fig. 5.2), the presence of FL-ASOct1 construct enhanced IL-5 promoter construct activities by 1.3 to 5.4-fold, depending on the stimulation method. These results suggest that the constitutive DNA binding of Oct1 factor has a repressive effect on gene expression.

5.2.1.2 Effect of Antisense Oct2 on IL-5 CLE0

The presence of antisense Oct2 increased the C/T-IL-5 construct activity by more than 3-fold after stimulation, but only 1.5-fold when the cells were stimulated with PMA/cAMP (Fig. 5.3C). A similar trend was also observed with the 509bp-IL-5 promoter construct (Fig. 5.3A). With the G/C/T-IL-5 construct, Oct2 antisense had a greater effect with PMA/cAMP than other stimulations (Fig. 5.3B).

Our group has subsequently determined through EMSA experiments that Oct2 does not bind to IL-5 CLE0 under PMA/cAMP stimulation (Schwenger et al., 2002). Importantly, as binding depends on the method of cell stimulation, these results suggest that Oct2 could not be the rate-limiting factor of IL-5 CLE0 activity.
A) 509bp-IL-5 promoter

B) G/C/T-IL-5 promoter
Figure 5.2: Effect of full-length antisense Oct1 expression construct on the A) 509bp-IL-5 promoter, B) G/C/T-IL-5 promoter and C) C/T-IL-5 promoter in PER117 cell line. pSI or FL-ASOct1 vector was co-transfected with tested construct, and transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
A) 509bp-IL-5 promoter

B) G/C/T-IL-5 promoter
Figure 5.3: Effect of full-length antisense Oct2 expression construct on the A) 509bp-IL-5 promoter, B) G/C/T-IL-5 promoter and C) C/T-IL-5 promoter in PER117 cell line. pSI or FL-ASOOct2 vector was co-transfected with tested construct, and transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
5.2.2 Effect of Short-length Antisense JunD and Fra2 on IL-5 CLE0

This section investigates the importance of the JunD and Fra2 transcription factors by reducing their expressions in PER117 cells, and observing the effects on the 509bp-IL-5, G/C/T-IL-5 and C/T-IL-5 promoters.

5.2.2.1 Effect of Antisense JunD on IL-5 CLE0

The presence of SL-ASJunD construct appeared to increase the activities of the IL-5 promoter constructs, suggesting a JunD-related repressive effect. This effect, however, was not as significant as those conferred by Oct1 and Oct2 transcription factors; the increases in activities of both IL-5 promoter constructs were only 2-fold or less (Fig. 5.4). The result suggests the presence of pre-existing JunD mRNA, which has been observed by our group in RT-PCR experiments (Schwenger et al., 2002), is not affected by antisense JunD.

5.2.2.2 Effect of Antisense Fra2 on IL-5 CLE0

Unlike the effect of antisense Oct1, antisense Oct2 or antisense JunD, the presence of antisense Fra2 reduced the activities of IL-5 promoter constructs across all types of cell stimulation. In unstimulated cells, antisense Fra2 did not affect C/T-IL-5 construct activity; this was expected as Fra2 transcription factor does not to bind IL-5 CLE0 in unstimulated PER117 cells (Schwenger et al., 2002) (see also Fig. 1.3). A 70% reduction of C/T-IL-5 promoter activity was observed in both PMA and PMA/cAMP-stimulated cells, whereas activity was reduced by 40% in PMA/CaI-stimulated cells (Fig. 5.5B). A similar trend was also observed on the activities of the 509bp-IL-5 promoter (Fig. 5.5A). These results suggest that antisense Fra2 inhibits the newly synthesized Fra2 mRNA, as it appears only after cell stimulation (Schwenger et al., 2002). Thus, Fra2 is the rate-limiting factor of hIL-5 CLE0 activity.
Figure 5.4: Effect of short-length antisense JunD expression construct on the A) 509bp-IL-5 promoter and B) C/T-IL-5 promoter in PER117 cell line. pSI or SL-ASJunD vector was co-transfected with tested construct, and transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
Figure 5.5: Effect of short-length antisense Fra2 expression construct on the A) 509bp-IL-5 promoter and B) C/T-IL-5 promoter in PER117 cell line. pSI or SL-ASFra2 vector was co-transfected with tested construct, and transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
5.3 Discussion

When antisense expression constructs of CLE0-binding transcription factor were co-transfected with IL-5 promoter constructs in PER117 cells, only antisense Fra2 reduced hIL-5 promoter activity (Fig. 5.5). The presence of antisense Oct1 increased reporter gene expression (Fig. 5.2), suggesting that the constitutive binding of Oct1 to CLE0 represses hIL-5 gene expression. This concurs with previous studies that showed constitutive binding of Oct1 to be inhibitory as mutations of the Oct1 sequence induced reporter gene activity more vigorously (Mori et al., 2000b; Mori et al., 1999b).

The presence of antisense Oct2 also did not inhibit reporter gene activity (Fig. 5.3), suggesting that Oct2 is not an important factor for hIL-5 production. This result follows the observations that binding of Oct2 to IL-5 CLE0 is not necessary for IL-5 transcription (Mordvinov, VA, unpublished data), and that Oct2 does not bind to IL-5 CLE0 under PMA/cAMP stimulation (Schwenger et al., 2002). However, deletion of Oct2 sequence from the IL-5 CLE0 element greatly reduced promoter activity (Mordvinov et al., 1999b). This inhibition might be due to the overlapping of the Oct2 binding sequence with the AP1 binding sequence (Thomas, Mordvinov & Sanderson, 1999). Deletion of the Oct2 sequence would also delete part of the AP1 sequence and thus, stopping AP1 transcription factors to bind.

The presence of pre-existing JunD mRNA before stimulation has been observed by our group in RT-PCR experiments (Schwenger et al., 2002). However, JunD protein production is inhibited by cycloheximide (CHX), which indicates that new translation of the protein is occurring (Schwenger et al., 2002). Thus, the ineffectiveness of antisense JunD (Fig. 5.4) shows an antisense effect on the newly translated JunD protein, but not on pre-existing protein, which will still be able to bind CLE0 and allow for hIL-5 transcription.

Fra2 protein production is inhibited by cycloheximide (CHX), and Western blotting experiments indicate that Fra2 protein appeared later than JunD protein after cell stimulation (Schwenger et al., 2002). The inhibitory effect of antisense Fra2 may
then act on the newly synthesized Fra2 protein. However, in contrast to JunD mRNA, Fra2 mRNA is not present before cell stimulation (Schwenger et al., 2002). Taken together with the effect of antisense Fra2 (Fig. 5.5), these results suggest that the synthesis of Fra2 protein is the rate-limiting factor of hIL-5 CLE0 activity.

It is not clear why antisense Oct and JunD constructs increased reporter gene expression. In the case of Oct1, it may be because this constitutively expressed factor is a repressor of expression (Mori et al., 2000b; Mori et al., 1999b). However, this explanation is unlikely in the cases of Oct2 and JunD. It is important to note that no direct tests were carried out to determine whether the antisense constructs were decreasing the target protein levels.

Glucocorticosteroids (GCS), therefore, remain the most effective anti-eosinophilic drugs available (Barnes, Pedersen & Busse, 1998). However, they also affect other genes such as IL-1, IL-2, IL-3, IL-4, IL-6, IL-8, IL-11, IL-12, IL-13, TNF-α, GM-CSF, RANTES, and ICAM-1 (Barnes, 1996); thus the need to identify a more specific target within the IL-5 gene. As CLE0 by itself was able to induce reporter gene expression (see Chapter 3), and was shown to be a powerful activator, the following chapter will determine the glucocorticoid target element, and to study the effect of dexamethasone on hIL-5 CLE0.
CHAPTER 6

EFFECT OF DEXAMETHASONE ON IL-5 CLE0
6.1 Experimental Approach

Despite the wide usage of glucocorticoids and the evidence of their effectiveness in the suppression of IL-5 expression, their mode(s) of action is still not clear. The effects of glucocorticoids are exerted by its binding to a single glucocorticoid receptor (GR) localized in the cytoplasm, which is able to translocate to the nucleus and act as a transcription factor (Adcock, 2001). Homodimeric GRs bind DNA consensus sites, termed glucocorticoid response elements (GREs), however, computer analysis has determined that the hIL-5 gene regulatory sequence contains no known GRE motifs (Mordvinov VA, unpublished data). The purpose of the following studies was to determine the element within the hIL-5 promoter by which dexamethasone exerted its effect. Identification of this target element will aid further research into the mode of action of dexamethasone on IL-5 gene expression.

To determine the dosage of dexamethasone to be used in this study, experiments were designed where increasing concentrations of dexamethasone were added to PER117 cells transfected with either 2045bp-IL-5 promoter (Fig. 6.1A), 1281bp-IL-5 promoter (Fig. 6.1B), 509bp-IL-5 promoter (Fig. 3.1A), or C/T-IL-5 promoter construct (Fig. 3.1C). PER117 and Jurkat cells were then transiently transfected with 509bp-IL-5 promoter, G/C/T-IL-5 promoter or C/T-IL-5 promoter, and pre-incubated for 10 minutes with the pre-determined dexamethasone dose before being stimulated (Section 2.10.3).
A) 2045bp-IL-5 promoter construct

B) 1281bp-IL-5 promoter construct

Figure 6.1: The 2045bp-IL-5 and 1281bp-IL-5 promoter constructs, created by Ms. D. L. Urwin using pGL3-Basic vector as backbone vector. Constructs are not drawn to scale.
6.2 Results

6.2.1 Effect of Dexamethasone on Endogenous hIL-5 Production

Initially, a preliminary experiment was designed to determine the effect of dexamethasone on endogenous IL-5 production by PER117 cells (Fig. 6.2), and it was easily seen that the reduction in IL-5 production is dependent on the dexamethasone concentration. Indeed, a dexamethasone concentration as low as 50nM could effectively reduce IL-5 production. This reduction, however, was dependent on the type of cell stimulation; at 50nM, dexamethasone reduced IL-5 production by nearly 100% in PMA/cAMP-stimulated PER117 cells, and by only 40% in PMA/Cal-stimulated cells (Fig. 6.2).

6.2.2 Dexamethasone Dose Response of hIL-5 Promoter Constructs

A series of deletion experiments were performed to observe the effect of a range of dexamethasone concentrations on different lengths of hIL-5 promoter. A similar pattern of dexamethasone effects could also be seen on the activities of the luciferase reporter gene driven by the 2045bp-IL-5 (Fig. 6.3A), the 1281bp-IL-5 (Fig. 6.3B), the 509bp-IL-5 (Fig. 6.3C) and the C/T-IL-5 (Fig. 6.3D) promoter constructs in PMA/cAMP-stimulated PER117 cells. Promoter construct activities were observed to be unaffected in PMA/Cal-stimulated cells. Thus, as all these constructs responded to dexamethasone in a similar manner to the C/T-IL-5 construct, it seems likely that hIL-5 CLE0 may be the target element for dexamethasone action.

The same low dosage of 50nM of dexamethasone was also observed to be effective in reducing reporter gene activities in PMA/cAMP-stimulated PER117 cells, whilst having no effect on reporter construct activities in cells stimulated with PMA/Cal. This dosage (50nM) was used in subsequent experiments to determine if hIL-5 CLE0 is the dexamethasone target element within the proximal 500bp region of the hIL-5 promoter.
A) 2045bp-IL-5 promoter

- U/S
- PMA/cAMP
- PMA/CaI

Luciferase activity (cps)

Dexamethasone (nM)

B) 1281bp-IL-5 promoter

- U/S
- PMA/cAMP
- PMA/CaI

Luciferase activity (cps)

Dexamethasone (nM)
Figure 6.3: Dexamethasone dose response of hIL-5 promoter constructs in PER117 cells. Transfection experiments were performed as described in Section 2.10. U/S denotes unstimulated cells.
Figure 6.2: PER117 endogenous dexamethasone dose response. IL-5 production was measured using IL-5 bioassay (Section 2.10.5) and is presented as relative to standard amounts of IL-5.
6.2.3 Effect of Dexamethasone on Short hIL-5 Promoter Constructs

Following the dexamethasone dose-response experiments, the effect of 50nM dexamethasone on hIL-5 promoter constructs was observed in PER117 cells subjected to all methods of stimulation. In PMA-stimulated cells, the activity of C/T-IL-5, G/C/T-IL-5 and 509bp-IL-5 promoters were reduced by 76% (Fig. 6.4C), 75% (Fig. 6.4B) and 94% (Fig. 6.4A), respectively. The levels of reduction of the C/T-IL-5 and G/C/T-IL-5 promoter construct activities reached 90% or more when cells were stimulated with PMA/cAMP and PMA/CD28 (Figs. 6.4B-C).

Furthermore, under PMA/Cal stimulation, the addition of 50nM dexamethasone had no effect reporter gene activities. This agrees with previous observations that dexamethasone is ineffective in reducing either IL-5 production or IL-5 promoter activity in PMA/Cal-stimulated cells. Most importantly, as the C/T-IL-5 promoter construct was as highly affected by dexamethasone as the 509bp-IL-5 promoter construct, the results indicated that hIL-5 CLE0 is the likely target for dexamethasone action in reducing hIL-5 production.

In Jurkat cells, as expected, the transfection experiments yielded a significantly lower luciferase expression (Fig. 6.5). Surprisingly, however, dexamethasone-treatment of these cells did not have any effect on reporter gene activity. As there have been no references to the ATCC TIB-152 Jurkat cell-line being a dexamethasone-resistant cell line, this result indicates that while this cell line is appropriate for inclusion as a negative cell line for IL-5 production, it lacks important elements that allow dexamethasone to exert its effect.
A) 509bp-IL-5 promoter

- Control
- Dex-treated

B) G/C/T-IL-5 promoter

- Control
- Dex-treated
C) C/T-IL-5 promoter

Figure 6.4: Effect of dexamethasone on the A) 509bp-IL-5 promoter, B) G/C/T-IL-5 promoter and C) C/T-IL-5 promoter constructs in PER117 cell line. Transfection experiments were performed as described in Section 2.10, and cells were pre-incubated for 10 minutes with 50nM dexamethasone before stimulation. U/S denotes unstimulated cells.
Figure 6.5: Effect of dexamethasone on the A) 509bp-IL-5 promoter, B) G/C/T-IL-5 promoter and C) C/T-IL-5 promoter constructs in Jurkat cell line. Transfection experiments were performed as described in Section 2.10, and cells were pre-incubated with 50nM dexamethasone before stimulation. U/S denotes unstimulated cells.
6.3 Discussion

Glucocorticosteroids (GCS) have remained the most effective anti-eosinophilic drugs available (Barnes, Pedersen & Busse, 1998). However, there is no evidence of a consensus GRE in the 2kb upstream region of hIL-5 (Mordvinov, VA, unpublished data). Evidence has been presented showing that NFAT binding to the −110 element was abolished by dexamethasone (Quan, McCall & Sewell, 2001), although it was unclear whether this was responsible for hIL-5 blockade.

The availability of deletion constructs from 2045bp-IL-5 down to C/T-IL-5 provided an opportunity to map the corticosteroid target. All the constructs tested under PMA/cAMP stimulation were inhibited by dexamethasone and had similar dose response curves (Fig. 6.3). On the other hand, cells stimulated with PMA/CaI were resistant (Fig. 6.3). Most importantly, the C/T-IL-5 construct was equally suppressed by dexamethasone as the 509bp-IL-5 construct (Fig. 6.4), identifying IL-5 CLE0 element as the primary target for dexamethasone. Furthermore, binding of transcription factors to CLE0 was inhibited by dexamethasone when using PMA/cAMP but not when using PMA/CaI to activate the cells (Schwenger, GTF, unpublished data); yet, dexamethasone did not significantly reduce the induction or the protein levels of the transcription factors (Kok, CC, unpublished data).

Quan and colleagues, however, proposed the REII region (position −123 to −92) of the hIL-5 promoter to be the main target for dexamethasone due to the sensitivity of protein-DNA binding in that region to the glucocorticoid (Quan, McCall & Sewell, 2001). CLE0 had been proposed to be the on/off switch for hIL-5 gene (Naora et al., 1994; Gruart-Gouilleux, Engels & Sullivan, 1995; Thomas, Mordvinov & Sanderson, 1999); thus, while dexamethasone might have an effect on REII, results from the C/T-IL-5 construct (Fig. 6.4) indicated CLE0 to be the more important target for dexamethasone.

With no GRE motifs present in the regulatory sequence, the sensitivity of hIL-5 production to dexamethasone further enforced the possibility that dexamethasone exerts its effect indirectly (without DNA binding) on the hIL-5 gene. The inhibitory
effect of dexamethasone may then be due largely to an interaction between activated GR and CLE0-binding transcription factors. Using PER117 cells nuclear extracts taken at 6 hours, other work from this group showed that dexamethasone had no effect on the binding of AP-1, Oct1 or Oct2 factors to CLE0 (Kok, CC, unpublished data). Thus it appeared unlikely that dexamethasone had a direct effect on the binding of any of these factors to the CLE0 element.

This difference provided a tool to investigate the mechanism of inhibition. The GILZ protein seemed a likely candidate as it is expressed in lymphoid cells (D'Adamio et al., 1997) and has been shown to interact with AP1 (Mittelstadt & Ashwell, 2001). This study showed that dexamethasone inhibited reporter gene activity under all types of stimulation except PMA/Cal (Fig. 6.3 and Fig. 6.4). Using RT-PCR, GILZ was found to be expressed in dexamethasone treated cells stimulated with PMA/Cal but not with stimulation by PMA/Cal (Kok CC, unpublished data), thus correlating with the inhibition of hIL-5 production.

Ectopic expression of GILZ inhibited hIL-5 transcription in the absence of dexamethasone, regardless of the stimulation conditions used (Kok, CC, unpublished data). These data are consistent with a role for GILZ in the blockade of hIL-5 transcription by corticosteroids. Interestingly, no direct interactions between GILZ and AP-1 were observed during maximal transcription of IL-5 (Kok, CC, unpublished data), indicating a secondary effect of GILZ maybe involved in inhibiting hIL-5 production. This finding concurred with the observation made by König and colleagues (König et al., 1992); who noted that direct interaction between GILZ and AP-1 could not be demonstrated by supershift experiments with an anti-GILZ antibody.
CONCLUSIONS
One of the main factors restricting studies of hIL-5 expression has been the limited availability of human T-cell lines that express IL-5 in an inducible fashion (Section 1.6.2). Several human tumorigenic cell lines have been used in IL-5 gene regulation studies, but they have disadvantages that led to the use of PER117 cell line in this study. The human T-cell leukemia cell line ATL-16T constitutively expresses IL-5 (Yamagata et al., 1995), but the mechanism behind this abnormal activation of the IL-5 gene is not known. The human HSB-2 cell line provides a model for inducible IL-5 gene regulation as IL-5 expression is inducible with PMA and ionomycin, and mRNA can be monitored by nuclear run-on using PCR (Rolfe & Sewell, 1997). Manipulation of this cell line in our laboratory, however, produces only very low amounts of IL-5 (Sanderson, C. J.; unpublished data). Human Jurkat cells have been used for the study of IL-4 regulation, but none of the clones available in this laboratory produced IL-5 under any conditions of stimulation. Jurkat cells are used in this study to study the ability of hIL-5 CLE0 to activate an IL-4 minimal promoter.

PER117 cell line is a prothymocyte cell line established from bone marrow cells of an eighteen-month old baby with acute lymphoblastic leukaemia (Kees et al., 1994). This cell line was chosen from many tested that expressed IL-5. It inducibly expresses IL-5 and IL-4 and, as in primary T-cells, there is no detectable constitutive expression of IL-5 (Kees et al., 1994). Expression of IL-5 can be induced with PMA and further enhanced by cAMP, Ca2+ and α-CD28 antibodies (Mordvinov et al., 1999a). Furthermore, IL-5 production can be inhibited by dexamethasone and cyclosporin A. These properties mimic the regulation of IL-5 observed in vivo, making this cell line a good model for this study.

The 5′-flanking region of the IL-5 RNA initiation site contains the GATA and CLE0 regulatory elements located immediately upstream of the TATA-box (Fig. 1.5). The high species conservation of IL-5 proximal promoter elements (Fig. 1.4), compared with the low conservation of other elements within the -509/+44 IL-5 promoter region, suggests an important role for GATA and CLE0. This study shows that hIL-5 CLE0 is a powerful activator as, together with the TATA box, it gives strong inducible expressions in PER117 cells, primary human T-cells and Jurkat cells (Chapter 3). When moved upstream of its normal position, hIL-5 CLE0 is also able
to induce the expression of an IL-4 minimal promoter in PER117 and Jurkat cell lines (Chapter 4).

Using an antisense approach that has been used previously to block GATA-3 expression (Finotto et al., 2001), this study also showed that only antisense Fra2 has an inhibitory effect on the expression of IL-5 (Chapter 5). Thus, it is proposed that Fra2 might be the rate-limiting transcription factor in IL-5 expression in PER117 cells.

The observation that the presence of antisense for other CLE0-binding factors increased reporter gene expressions suggests that the antisense constructs were not inactive; however, further experiments are required to ensure the effectiveness of the antisense constructs. Initially, RT-PCR experiments should be employed to make certain the antisense mRNA of each transcription factor is produced by the constructs. Northern hybridisation experiments should then follow to determine the amount of antisense mRNA produced, allowing adjustments, if needed, of the amount of antisense constructs to be co-transfected with the hIL-5 constructs. After co-transfection of the antisense constructs with the hIL-5 constructs, protein levels of each transcription factor should then be compared to wild-type protein levels through Western Blotting experiments. Time, however, did not allow for these follow-up experiments to be carried out.

The antisense results of this study (Chapter 5) agrees with observations made concurrently by this laboratory (Schwenger et al., 2002). RT-PCR experiments have shown that JunD mRNA is present in unstimulated cells. This constitutively expressed mRNA, however, is not translated as subsequent Western Blotting experiments showed that no JunD protein is detectable in the cytoplasm, and that the protein is present in the nucleus only after cell stimulation. The same experiments showed that de novo synthesis of Fra2 is required as the mRNA is not present in unstimulated cells and the protein appears in the cytoplasm and the nucleus after cell stimulation. Thus, it can be suggested that by inhibiting the production of Fra2, the expression of IL-5 will be reduced. Fra2, however, does not only control the production of IL-5; it is also an important factor in the regulation of several other genes, including intercellular adhesion molecule-1 (ICAM) (Farina et al., 1997),

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clusterin (Jin & Howe, 1999), collagenase-3 (Varghese, Rydziel & Canalis, 2000) and utrophin (Galvagni, Cantini & Oliviero, 2002). Hence, it is not clear whether Fra2 is likely to be a useful drug target for inhibiting IL-5 production, as the side effects maybe unacceptable.

Glucocorticosteroids (GCS) remain the most effective anti-eosinophilic drugs available (Barnes, Pedersen & Busse, 1998). Computer analysis showed that no glucocorticoid response element (GRE) motifs were present in the hIL-5 regulatory sequence (Mordvinov, VA; unpublished data). Deletion constructs of the hIL-5 promoter region indicated that dexamethasone exerts its effect within the 500bp region (Chapter 6). This study shows a hIL-5 promoter fragment containing the CLE0 element alone responded to dexamethasone treatment in a similar manner to the 500bp region (Chapter 6), identifying IL-5 CLE0 as the primary target for dexamethasone in PER117 cell line.

Work of this laboratory has indicated that dexamethasone had an indirect effect on CLE0-binding transcription factors (Kok, CC; unpublished data). The inhibitory effect of dexamethasone may be due largely to an interaction between activated GR and genes whose products can directly interfere with a CLE0-binding transcription factor, such as the glucocorticoid-induced leucine zipper (GILZ) factor (Ayrolidi et al., 2001; Mittelstadt & Ashwell, 2001). Further work of this laboratory then indicated GILZ is acting through the AP1 proteins binding to the CLE0 element (Kok, CC, unpublished data). However, no direct interactions between GILZ and AP-1 were observed during maximal transcription of IL-5 in PER117 cells (Kok, CC, unpublished data), indicating a secondary effect of GILZ maybe involved in inhibiting hIL-5 production (Konig et al., 1992).

Glucocorticosteroids also affect other genes such as IL-1, IL-2, IL-3, IL-4, IL-6, IL-8, IL-11, IL-12, IL-13, TNF-α, GM-CSF, RANTES, and ICAM-1 (Barnes, 1996). Thus, as GILZ expression appears to be limited to lymphocytes, it may be a potential target for anti-allergy drugs. Non-steroidal activators of GILZ may provide activity against allergy with fewer side effects that those produced by using corticosteroids. These possibilities open up new research areas whereby the actions of dexamethasone on the regulation of hIL-5 production can be further investigated.
While PER117 cell line can be used as a first approach to study IL-5 regulation, it will be important to follow up with primary T-cells. Studies with primary T-cells, however, is limiting because of transfection inefficiencies and difficulties in obtaining un-degraded nuclear extracts (Sanderson et al., 1999). These limitations prove to be the drawback of this study, as time did not allow for the optimisation of the conditions required.

The high species conservation of IL-5 proximal promoter elements (Fig. 1.4), compared with the low conservation of other elements within the -509/+44 IL-5 promoter region, suggests an important role for GATA and CLE0. Alignment of human, mouse and rat genomic sequences by Debra Urwin has identified at least two distal conserved elements (Urwin, 2004). DNaseI footprint experiments implicate the region between positions -6287 to -6205 as the functional element in the upstream conserved region (UCR)-6.5 enhancer. This sequence was shown to be 80% conserved across the human, mouse and rat species, and contains consensus-binding sites for the transcription factors Oct1, AP1 and GATA-3 (Urwin, 2004). The high species conservation of both the proximal and the upstream enhancer regions, as well as the similar transcription factors binding to the functional elements within the regions, indicate that IL-5 gene regulation is specific, and that this specificity can be exploited in finding compounds which can regulate the production of IL-5 in eosinophilic diseases.
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