Characterization of a Mast Cell Specific Enhancer Located within the Interleukin-4 Gene

by

Gregory W. Henkel

A Dissertation

presented to the Department of Microbiology and Immunology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

April 1994

APPROVED:

..... [redacted] .

(Professor in Charge of Thesis)

..... [redacted] .....

(Chairman, Graduate Council)

TABLE OF CONTENTS

Collection of Papers

FIGURES

I. Manuscript #1

II. Manuscript #2

Acknowledgments

I would first like to thank God for the numerous gifts I have received and for the many friends I have made since I have been at OHSU. I wish to thank Melissa Brown for her guidance, her honesty, her patience and her friendship. I also want to thank the committee members, Miles Wilkinson, David Pribnow, David Henner, Maggie So and Rob Moses, for their time to read this thesis and for their input during the course of this study.

Because of the wonderful people in the Brown, Henner and Segal lab it made the work go by in a more enjoyable manner. I'll never forget the wine-tastings, gatherings at the pub, various dinner parties, skiing at Mount Hood or kayaking down the Deshutes river- I had a blast. I would especially like to thank David Tara who among other things got me started on many of the techniques in the lab and more importantly introduced me to fly fishing. I want to thank all the friends I have made for their support, humor, wisdom and companionship, especially, to the dudes I hung out with the most: my California buds, Mark Hansen and Amanda Roberts, and my Iowa bud, Jane Richards.

I want to thank my family for their love and encouragement, especially my brother Gary, who spent alot of time up here with me. Now for the greatest person in my life, who stood by me for these last 4 1/2 years, owns stock in MCI, logged alot of frequent flyer mileage and was very patient and understanding. I not only want to thank my wife Marian for her love, but also dedicate this thesis to her- I love you sweetpea! I am coming home.

Abstract

Interleukin-4 is an important immunoregulator that was originally defined as a T-cell cytokine. However, within the last seven years it has been demonstrated that mast cells and basophils also express this cytokine.

Mature mast cells are non-circulating and are found in variety of highly vascularized tissue locations e.g. skin, lungs and intestines. The production of IL-4 by these cells may have distinct effects in local inflammatory reactions relative to T-cell IL-4 production. Although there have been several published studies defining transcriptional components that regulate IL-4 in T-cells, there has been no such work reported in mast cells.

Several long-term mouse mast cell lines have been used in this study to examine the regulatory mechanisms that control IL-4 gene expression in these cells. DNAse I hypersensitivity analysis was used to begin to identify *cis*-regulatory elements that control IL-4 transcription in mast cells. Based on this analysis, a hypersensitive site (hs) was localized to the second intron of the IL-4 gene in nuclei isolated from two transformed mast cell lines that constitutively express this cytokine. This hs site was not found in T-cells that express IL-4 upon induction with a phorbol ester. A 683 base pair fragment from the hs region was isolated and subcloned into an SV40 or IL-4 promoter/CAT reporter plasmid. The IL-4 intronic fragment demonstrated prototypic enhancer activity in transient transfection assays in transformed and non-transformed mast cells but not T or B-cells.

To identify critical *cis*-acting elements that regulate this enhancer, serial deletions from either the 5' or 3' end of the 683 base pair fragment

were generated. Their effect on enhancer activity was assessed in transient transfection assays. Two functionally independent subregions (E1 and E2) were defined based on this analysis. Previously described protein binding sites for SP1 and GATA transcription factors were identified by sequence analysis in the E1 subregion. An ets binding site was located in the E2 subregion. Site-directed mutagenesis of these protein binding sites demonstrated that both the GATA and ets binding site, but not the SP-1 sites, were critical for enhancer activity. Specific proteins that bind to the IL-4 GATA or ets binding site were detected in several mast cell nuclear extracts by mobility shift assays. The use of antibodies in combination with mobility shift assays identified the mast cell factors associated with the IL-4 GATA sequence as GATA-1 and GATA-2. Antibodies to PU.1, an ets family member, demonstrated that this factor was binding to the IL-4 ets site. GATA-1, GATA-2 and PU.1 are restricted to distinct cell-types including mast cells, but not T-cells. Therefore, it is likely that these factors are important in regulating the lineage restricted activity of the IL-4 intronic enhancer in mast cells.

Introduction and Literature Review

Interleukin 4 (IL-4) belongs to the ever expanding family of secreted protein factors called cytokines. Cytokines function as mediators in cell to cell communication within relatively short distances and act at very low concentrations. Most cytokines are pleiotropic in terms of their biological activities and target cells. They primarily regulate immune function *eg.* growth, activation, differentiation, cell trafficking, as well as the development of immune cells during hematopoiesis. Cytokines are proposed to function in a networking fashion in which several different cytokines acting in combination at the cell surface elicit a particular response. Thus, the types of cytokines expressed as well as their local concentration will be important in the developing immune response. The regulation of cytokine gene expression is, therefore, a critical point in establishing the correct "messages" needed to direct the immune response.

IL-4 is a potent immunoregulatory cytokine that was originally shown to be expressed by T-helper cells (Th) (Howard et al., 1982). The primary production of IL-4 is in a certain subpopulation of Th cells. In both mouse and humans the Th population of cells can be subdivided into at least three distinct phenotypes based on their cytokine profile (Firestein et al., 1989; Mosmann et al., 1986; Mosmann and Coffman, 1989; Romagnani, 1991). Two of the phenotypes express the same but also, mutually exclusive sets of cytokines. The Th1 phenotype is distinguished by its production of IL-2 and IFN-γ, while the signature cytokines produced by Th2 cells are IL-4, IL-5, IL-6 and IL-10 (Mosmann and Coffman, 1989). The Th0 phenotype has an unrestricted cytokine profile,

including expression of IL-2, IFN-γ and IL-4 (Firestein et al., 1989). Although the major portion of IL-4 production in T-cells is seen in the $CD4^+$ Th population, certain subpopulations of $CD8^+$ T- cells can also secrete IL-4 (Paliard et al., 1988; Salgame et al., 1991; Seder et al., 1992a).

There has been a plethora of *in vitro* and *in vivo* studies defining the biological functions of IL-4. Since its initial discovery in T-cells, the diverse activities of IL-4 in the immune response have been associated with T-cell production of this cytokine. In turn there have been several papers published defining and characterizing the elements that regulate transcription of the IL-4 gene in human and mouse T-helper cells (Abe et al., 1992; Bruhn et al., 1993; Li-Weber et al., 1992; Li-Weber et al., 1993; Szabo et al., 1993; Tara et al., 1993; Todd et al., 1993). However, there are other cell types that produce IL-4 that until recently, have been ignored. It has been appreciated within the last seven years that mast cells and basophils are also a major source of IL-4 as well as a variety of other cytokines (Galli, 1993). Both of these cell types are granulocytic and share several common characteristics (Galli, 1990). Mast cell and basophil secretion of histamine, prostaglandin D_2, leukotrienes, cytokines, and other mediators provide the effector functions for immediate hypersensitivity and other inflammatory responses (Galli, 1990). To date there have been no studies published examining the role of IL-4 in various immunological and inflammatory reactions associated with these cells. Likewise, there have been no studies to define the molecular events that control IL-4 gene expression in either mast cells and basophils.

The focus of this thesis work was to begin to define critical elements that regulate transcription of the IL-4 gene in mast cells. During this

work, an enhancer element was identified in the second intron of the IL-4 gene that appears to be mast cell specific. The two manuscripts generated from this study detail the initial discovery of this enhancer element and its molecular characterization. Two key *cis* and *trans*-acting elements were identified that not only regulate the enhancer function but may also be important in the cell-restricted activity of this element.

I. Mast Cells

A. Origin, Distribution and Heterogeneity

Mast cells are derived from circulating precursor cells originating from a multipotent stem cell in the bone-marrow (Kitamura et al., 1978; Kitamura et al., 1979; Kitamura et al., 1981). Mature mast cells are non-circulatory and are widely distributed throughout connective tissues adjacent to blood and lymphatic vessels, near nerves and beneath epithelial surfaces. In humans, mast cells can be found in such diverse places as the skin, lungs, gastrointestinal tract, lymphoid tissues, synovia, heart, pancreas, bone-marrow, adrenal glands, thalamus and hypothalamus (Enerback and Norrby, 1989; Galli, 1990; Theoharides, 1990). The different locations of mast cells enable them direct access to various environmental stimuli (Galli, 1990).

Early studies noted in mice and rats that mast cells from various anatomical locations exhibited differences in morphological characteristics (Enerback and Norrby, 1989; Galli, 1990). Histochemical staining was able to discern two subtypes of mast cells from the intestines and from the skin or peritoneal cavity. These cells are referred to as mucosal mast cells (MMC) and connective-tissue mast cells (CTMC), respectively (Enerback

and Norrby, 1989; Galli, 1990). Later, it was appreciated that these two types of mast cells also varied in mediator content, reactivity to external stimuli and function (Enerback and Norrby, 1989; Galli, 1990). For example, MMC are dependent on T-cell derived IL-3 for their proliferation. Athymic mice have a reduced number of MMC; however, administration of IL-3 to these mice induces MMC proliferation (Abe et al., 1988; Ruitenberg and Elgersma, 1976). In contrast, CTMC are T-cell independent and are found in equal numbers in both normal and T-cell deficient mice (Ruitenberg and Elgersma, 1976).

It is possible that there are distinct mast cell lineages that give rise to the two different phenotypes or there may be a single lineage that undergoes differentiation along different pathways governed by the local microenviroment. Many studies both *in vitro* and *in vivo* support the later possibility (Galli, 1990). Furthermore, these studies demonstrated that the phenotype of a mast cell is not fixed (Kitamura et al., 1987). Mast cells with MMC-like characteristics can be cultured from bone-marrow, spleen and fetal liver with the addition of IL-3 (Galli, 1990). Incubation of bone-marrow derived MMC (BMMC) on fibroblast monolayers without IL-3 results in a switch from MMC-like to CTMC-like phenotypic characteristics (Levi-Schaffer et al., 1986). This was also demonstrated with a cloned MMC line (Dayton et al., 1988). Addition of IL-3 to the fibroblast/BMMC co-cultures enhanced proliferation and histamine content in these CTMC-like cells (Levi-Schaffer et al., 1986). Fibroblast conditioned media could also support growth and transdifferentiation of MMC to CTMC-like phenotype (Jarboe et al., 1989). The soluble factor released by fibroblasts was found to be stem-cell factor (SCF), the ligand for *c-kit* (Denburg, 1992; Anderson et al., 1990; Williams et al., 1990).

Incubation of the MMC-like cells in the presence of both IL-3 and IL-4, without fibroblasts, also supports the transdifferentiation to CTMC-like phenotype (Hamaguchi et al., 1987). Other factors that can influence mast cell growth and/or differentiation in vitro include IL-9, IL-10 and nerve growth factor (Denburg, 1992).

A mast cell deficient mouse strain (W/W^{v}) has been very useful in studying mast cell differentiation/maturation in vivo. Mast cells can be reconstituted in these mice by transplanting bone-marrow derived cultures of mast cells from normal congenic littermates (Galli, 1987). The deficiency is due to a mutation in the W locus encoding the c-*kit* tyrosine kinase receptor (Geissler et al., 1988). Injecting bone-marrow derived MMCs (BMMC) i.v. into mast cell deficient mice gave rise to both MMC and CTMC in their appropriate anatomical locations (Nakano et al., 1987). On the other hand, injection into the peritoneal cavity resulted in production of only CTMC. Besides the MMC to CTMC switch, it was shown that CTMCs could also switch to MMC in the appropriate environment (Nakano et al., 1987). Injection of clonally developed CTMCs into either the stomach or skin of the mast cell deficient mice, resulted in production of CTMC in the connective tissue (skin and stomach muscle) and MMC in the stomach mucosa. Finally, a single CTMC was injected into the stomach wall (Sonoda et al., 1986). Mast cells of the MMC phenotype developed in the mucosa while CTMCs developed in the stomach muscle. Besides unilateral switching, other studies have shown that mast cells can transdifferentiate multiple times (Galli, 1990). These experiments support the hypothesis that phenotypically distinct mast cells develop from a common lineage and that their differentiation or transdifferentiation is dependent on the local microenviroment. The

different types of mast cells and their apparent plasticity may have functional significance in terms of health and disease. The flexibility in mast cell phenotype may allow these cells to adapt to changes within the local microenviroment due to injury, immunological or pathological responses (Galli, 1990).

Human mast cells isolated from different anatomical sites also display heterogeneity. The separation into mucosal and connective tissue subclasses, however, is not as clear in humans. The key differentiation marker for human mast cells is in their protease content (Schwartz, 1989). Some mast cells express only tryptase (MC^T e.g. lung and small intestine), while others express both tryptase and chymase (MC^{TC} e.g. skin and sub-mucosa of the small intestine). Interestingly, patients with hereditary or acquired T-cell deficiencies have a reduced number of MC^T in their intestines, suggesting that these type of mast cells may be T-cell dependent as the mouse/rat MMC (Galli, 1990).

B. Mediators and Mode of Activation

The most striking characteristic feature of a mast cell is the presence of large dense granules located in the cytoplasm. These granules contain various biological modifiers implicated in a wide variety of inflammatory and immunologic processes (Galli and Lichtenstein, 1988; Schwartz and Austen, 1984).

One of the well known mediators stored in mast cell granules is the biogenic amine, histamine. Rat mast cells also store the biogenic amine, serotonin. Histamine is one of the major proteins stored in mast cells and has various physiological effects including vasodilation, bronchial constriction, down-regulation of T-cell mediated cytotoxicity, lymphokine

release, and enhancing eosinophil-mediated complement-dependent damage of *Shistosoma* infection *in vitro* (Galli and Lichtenstein, 1988; Schwartz and Austen, 1984). Another major component found in mast cells are the glycoaminoglycans (GAG)- heparin or chondroitin sulfate. In the mouse, heparin is found in CTMC and chondroitin sulfate is found in MMC (Galli and Lichtenstein, 1988; Schwartz and Austen, 1984). The GAGs are the matrix proteins of the granules and help to package all the other preformed mediators in an orderly array (Galli and Lichtenstein, 1988; Schwartz and Austen, 1984). The well known function of heparin is its anticoagulant activity. It can also enhance fibronectin binding to collagen, inhibit eosinophil cationic protein, and modulate neutral proteases released from mast cells (Schwartz and Austen, 1984). The neutral proteases, including chymase, carboxypeptidase A and tryptase are a third major component in mast cells (Schwartz and Austen, 1984). Tryptase is found only in human mast cells. These proteases among other things regulate complement and kinin activation. Chemotactic factors for eosinophils and neutrophils are also stored in the mast cell granules (Schwartz and Austen, 1984). Besides preformed factors, activated mast cells make several newly formed mediators primarily through the arachidonic acid metabolic pathway. These arachidonic acid derived mediators include the prostaglandins and the leukotrienes (Galli and Lichtenstein, 1988). Many of these factors play a role in augmenting vasopermeability and bronchial constriction. Leukotriene LTB_4 enhances neutrophil chemotaxis and adherence. Prostaglandin (PGD_2) can inhibit platelet aggregation while TXA_2 leukotriene enhances platelet aggregation (Galli and Lichtenstein, 1988).

Mast cells normally possess a large number of IgE molecules bound to their cell surface receptor $F_c\varepsilon RI$ (Enerback and Norrby, 1989). The

IgE molecule in turn can bind multivalent antigens that lead to bridging of the IgE receptors, initiating degranulation (Ishizaka and Ishizaka, 1984). The physiochemical features of the interactions between the mediators and the granule matrix allows for a controlled release that regulates the local bioavailability of these mediators (Enerback and Norrby, 1989). Mast cells also express F_C receptors for IgG molecules which when occupied by immune IgG complexes can activate mast cells (Enerback and Norrby, 1989). There are also other non-immunological secretagogues that can stimulate mast cell degranulation, including ATP, kallidrein, kinins, major basic protein from eosinophils, bee venom, cobra venom, curare, morphine and calcium ionophores. Neuropeptides such as somatostatin and substance P can activate mast cells as well as components of complement, C3a or C5a. Polyamines such as 48/80 and polylysine can activate mast cells by cross-linking $F_C\varepsilon RI$ (Enerback and Norrby, 1989). Finally, certain cytokines, such as MIP 1-α and SCF, can cause histamine release by mast cells. (Alam et al., 1992; Borish and Joseph, 1992; Coleman et al., 1993a). Because mast cells can respond to a variety of external stimuli suggests that they have a broad role in normal physiological as well as in immunological and pathological conditions.

C. Mast cells are a Major Source of Cytokines

The first indication that mast cells make cytokines came from studies with Abelson murine leukemia (AB-MuLV) virus transformed mast cell lines. It was shown that these transformed cells express GM-CSF but not IL-3 (Chung et al., 1986; Pierce et al., 1985). Other reports with AB-MuLV transformed cell-lines demonstrated constitutive production of IL-3, GM-CSF and IL-4 (Brown et al., 1987). In this same study, several IL-3

dependent mast cell lines derived from fetal liver demonstrated low levels of IL-4 mRNA but undetected product (Brown et al., 1987). However, stimulation with either a calcium ionophore or cross-linkage of the $F_c\varepsilon RI$ at the cell surface augmented the level of IL-4 mRNA and product, as well as, IL-3, IL-5 and IL-6 (Plaut et al., 1989). Further work with other IL-3 factor dependent mast cell-lines activated through the IgE receptor has expanded the list of cytokines secreted by mast cells: IL-1,-3,-4,-5,-6, TNF-α, GM-CSF, IFN-γ, MIP1 α/β, JE and TCA3 (Burd et al., 1989).

Freshly isolated mast cells also produce cytokines. Mast cells isolated from mouse peritoneal cavity were shown to store TNF-α in their cytoplasmic granules that was released upon IgE dependent activation. Also newly formed TNF-α was produced during activation of the cell (Gordon and Galli, 1991). Human mast cells isolated from skin, lung or nasal biopsies were also shown to store and secrete IL-4, IL-5, IL-6 and IL-8 (Bradding et al., 1992; Bradding et al., 1993). A population of non-T/B cells can be cultured from human bone marrow. Cross-linkage of $F_c\varepsilon RI$ with anti-IgE resulted in expression of both IL-4 and IL-5 that could be enhanced with the addition IL-3 (Piccinni et al., 1991). Sorting by $F_c\varepsilon RI$ expression demonstrated that $F_c\varepsilon RI^+$ cells were responsible for expressing IL-4 and IL-5. Histochemical analysis identified this population of cells as a mix of mast cells and basophils, with a higher proportion of mast cells (Piccinni et al., 1991). A similar subset of IL-4 producing cells was identified from mouse spleen and bone-marrow (Ben-Sasson et al., 1990). In this case, a higher proportion of the cells were basophils and a very small percentage were of mast cell origin (Seder et al., 1991).

D. Re-evaluation of Mast Cell Functions

Mast cells were first identified as the prime effector cells in the IgE dependent immediate-hypersensitivity response associated with anaphylactic reactions to certain foods, insect venom, pollens and many different types of allergens (Enerback and Norrby, 1989). Many of these acute reactions are due to the sudden release of the various preformed and newly formed mediators secreted by mast cells. Although mast cells are most noted for their effects in the immediate hypersensitivity response, it has been demonstrated, using mast cell deficient mice, that these cells may play a protective role against certain intestinal helminth infections and dermal tick infections (Galli, 1993). Besides their role in acute inflammation, mast cells have also been implicated in chronic disorders such as rheumatoid arthritis and scleroderma (Irani et al., 1992; Mican and Metcalfe, 1990). Because mast cells have been shown to express cytokines, their role not only in the immediate-hypersensitivity response but in other acute and chronic inflammatory conditions must be re-evaluated.

The cytokines secreted by mast cells not only can regulate immunological responses, but are important in the inflammatory response, tissue-repair, hemostasis and hematopoiesis (Galli, 1993). Mast cells have been implicated in all of these processes (Galli, 1993). Besides their role in the acute phase of an allergic response mast cells also participate in more chronic allergic inflammatory reactions. Following the immediate-hypersensitive response (wheal) in many allergen induced individuals, a secondary response (flare) develops hours after the initial challenge (Borish and Joseph, 1992; Galli, 1993). This late-phase response (LPR) is associated with increased vasopermeability and infiltration of leukocytes (Borish and Joseph, 1992; Galli, 1993). Much of the morbidity associated with chronic allergic disorders such as asthma and atopic dermatitis is

believed to be due to the leukocytes infiltrating the affected tissue, rather than a consequence of the immediate release of mast cell mediators (Galli, 1993). Many of the cytokines produced by mast cells have been associated with allergic diseases. In contrast to the other mediators released by mast cells, cytokines are more potent inflammatory mediators and may have a longer half-life (Borish and Joseph, 1992). Cytokines such as IL-3, IL-5 and GM-CSF may contribute to the local accumulation and activation of eosinophils and monocytes/macrophages (Borish and Joseph, 1992). Mast cell numbers increase during most allergic responses (Borish and Joseph, 1992). Therefore, both IL-3 and IL-4 may serve as autocrine factors regulating mast cell growth. TNF-α appears to be important in the trafficking of leukocytes to the inflamed site. One group has demonstrated that TNF-α was important for approximately 50% of neutrophil infiltration during a late-phase response following mast cell activation (Wershil et al., 1991). In two separate studies, TNF-α from human mast cells was shown to augment expression of a leukocyte adhesion molecule (ELAM-1) on adjacent vascular endothelial cells (Klein et al., 1989; Walsh et al., 1991). Because adhesion molecules are important for cellular trafficking, these experiments further support a role for TNF-α in leukocyte infiltration during the inflammatory response.

Recruitment of leukocytes by mast cell cytokines may also be an important defense mechanism to protect against parasitic infection. IgE-activated eosinophils, macrophages and/or platelets are believed to be important effector cells against shistosomes and other parasitic diseases (Capron et al., 1986; Galli, 1993). For example, mast cell production of cytokines such as IL-4 and IL-5 may be important in the development of a immune response to *Shistosoma mansoni ie.* IL-4 induces IgE secretion and

IL-5 is important for eosinophilia. Recently it was demonstrated that an increased number of $F_c\varepsilon RI^+$ non-T/B cells could be isolated from *S. mansoni* infected mice compared to non-infected controls. These cells express IL-4 but very little IL-5 (Williams et al., 1993). The population of $F_c\varepsilon RI^+$ cells contained both mature mast cells and basophil-like cells.

Recruitment of leukocytes by mast cell cytokines may also play a role in chronic inflammatory conditions such as rheumatoid arthritis and scleroderma (Galli, 1993). Under controlled conditions, however, the inflammatory reaction itself is critical to the survival of higher organisms. Inflammation is the response of living tissue to injury of any sort: chemical, physical or microbiological (Iverson, 1989). During inflammation, a complex series of reactions occurs that prevents further tissue damage, destroys the infective organism and activates the repair process (Baumann and Gauldie, 1994). Mast cell deficient mice have been used to study the role of mast cells in the inflammatory reaction. Induction of tissue damage in mast cell deficient mice, either by chemical contact to the skin or formation of immune complexes intradermally, resulted in a decrease in tissue-swelling, leukocyte infiltration, fibrin deposits and a reduction in vascular permeability. These deficiencies were corrected by reconstitution of mast cells in the dermis of these mice (Ramos et al., 1992; Wershil et al., 1988; Zhang et al., 1992). Proinflammatory cytokines such as IL-1, IL-6, TNF-α are important in regulating the acute inflammatory response (Baumann and Gauldie, 1994). Besides monocytes/macrophages, mast cells express these cytokines and therefore, further support a role for these cells in the acute inflammatory reaction.

Due to the fixed locations of mast cells in various tissues, the local consequences of mast cell cytokine release is expected to be more

significant than any systemic effects (Gordon et al., 1990). Local release of mast cell cytokines can effect several different target cells *eg.* endothelial cells, fibroblasts, local and infiltrating leukocytes. These various target cells in turn may secrete the same and/or different types of cytokines, establishing a cytokine cascade that can sustain or amplify the reaction, and in normal situations, eventually down-regulate the inflammatory reaction (Galli, 1993).

Because mast cells can respond to various types of stimuli, it is possible that cytokine production may be differentially regulated and/or display distinct kinetics (Gordon et al., 1990). Distinct patterns of cytokine production may be important in regulating the various biological effects associated with mast cell activation. Therefore, it will be important to understand the molecular mechanisms that control cytokine production in mast cells. Because of its many diverse immunological activities, especially its effects on mast cell growth and function, we decided to focus on IL-4 production by mast cells.

II. IL-4 Demonstrates Diverse Biological Effects

A. B-cells

IL-4 was first defined *in vitro* as a co-stimulator of B-cell growth and differentiation (Howard et al., 1982). In support of its role as a growth factor for B-cells, it has been recently shown that IL-4 acting as an autocrine growth factor for a B-lymphoma cell-line that constitutively expresses this cytokine (Louie et al., 1993). On the other hand, IL-4 can also inhibit the IL-2 mediated growth and differentiation of B-cells (Jelinek and Lipsky, 1988). The mechanism behind this antagonistic effect appears

to be related to the ability of IL-4 to down-regulate the IL-2 receptor on B-cells (Karray et al., 1990; Lee et al., 1990).

IL-4 may influence the antigen presenting function of B-cells by its ability to induce increased expression of MHC class II (Paul, 1991). One of its more noted roles in regulating B-cell function is its ability to induce class-switching and subsequent expression of IgE and IgG1 (Coffman et al., 1986; Del Prete et al., 1988; Finkelman et al., 1990). As previously mentioned, IgE is important in activating both mast cells and basophils through cell surface receptor engagement. Other cells that potentially can respond to IgE include macrophages, monocytes, B-cells, T-cells, eosinophils and platelets. These cells express the low affinity IgE receptor $F_c\varepsilon RII$ (CD23) (Delespesse et al., 1992). IL-4 can up-regulate $F_c\varepsilon RII$ expression on T-cells, B-cells, monocytes/macrophages and eosinophils (Kawabe et al., 1991a; Kawabe et al., 1991b; Paul, 1991). IgE bound by B-cells may be important in expanding and/or sustaining IgE production, whereas, eosinophils, macrophages and platelets activated by IgE have demonstrated cytotoxic activity to certain parasites (Capron et al., 1986; Delespesse et al., 1992). $F_c\varepsilon RII^+$ cells are also associated with inflammatory reactions and allergies (Baumann and Gauldie, 1994; Borish and Joseph, 1992).

The ability of IL-4 to select for IgE and IgG1 secretion by B-cells has been verified *in vivo*. Mice treated with anti-IL-4 do not develop an increase in serum IgE levels normally associated with nematode infections or some related stimuli (Finkelman et al., 1986). Transgenic mice that overexpress IL-4 possess high serum levels of IgE and IgG1 relative to their normal littermates (Tepper et al., 1990). Finally, IL-4 knock-out mice demonstrated no detectable levels of IgE and significantly reduced

levels of IgG1 (Kopf et al., 1993). The levels of IgE in serum remained undetectable even after stimulation with anti-IgD or nematode infection, both potent inducers of IgE and IgG1. The serum level of IgG1 did increase upon stimulation but remained significantly less than the normal control littermates. Therefore, the regulation of IgG1 synthesis is not absolutely dependent on IL-4.

It is possible that mast cell secretion of IL-4 can modulate its own function by inducing IgE secretion by B-cells. In support of this hypothesis it was shown that co-cultures of LPS activated B-cells with non T/B cells in the presence of IL-3 or by IgE receptor cross-linkage could induce isotype switching to IgE and IgG1 (Ledermann et al., 1992). Addition of anti-IL-4 blocked the production of IgE by the B-cells (Ledermann et al., 1992).

B. T-cells

IL-4 is also a growth factor for T-cells. Resting T-cells can survive in culture without dividing in the presence of IL-4. However, co-addition of a phorbol ester with IL-4 induced approximately 50% of the cells to enter S phase and divide (Hu-Li et al., 1987). IL-4 can act as an autocrine growth factor for some long term Th2 cell-lines (Fernandez et al., 1988; Greenbaum et al., 1988; Kurt-Jones et al., 1987). Their growth in response to mitogens or antigen and antigen presenting cells (APC) can be inhibited with anti-IL-4 (Kurt-Jones et al., 1987). Some of these Th2 cell-lines required IL-1 as a co-stimulus for growth in response to mitogens or exogenous IL-4 (Kurt-Jones et al., 1987). In general, many of the long term Th2 cell lines do not respond well for extended periods to antigen and APCs alone; IL-2, the prototypic T-cell growth factor, is often

supplemented to sustain these cells (Paul, 1991). Therefore, the role of IL-4 as a long term growth factor is still questionable (Paul, 1991). Th1 cell-lines, on the other hand, respond to IL-2 and do not respond to IL-4 with or without IL-1 (Paul, 1991).

IL-4 appears to play a role in T-cell development in the thymus. IL-4 mRNA has been detected in thymocytes *in vivo* during fetal development (Carding et al., 1989). The level of expression is highest at day 15 but rapidly declines to where it is undetectable by day 18 of gestation and onward.

Analysis of thymocyte development in fetal thymic organ cultures demonstrated that incubation with IL-4, inhibited thymocyte growth in a dose-dependent manner (Plum et al., 1990). Treatment of 14 day thymic organ cultures with IL-4 also blocked T-cell differentiation. There was a significant decrease in $CD4^+CD8^+$ double positive (DP) T-cell population, an increase in the frequency of $CD4^-CD8^-$ double negative cells and a decrease in the frequency of both types of single positive (SP) cells ($CD4^+CD8^-$ and $CD4^-CD8^+$) relative to untreated thymic lobes. Treatment of thymic lobes isolated after 14 days of gestation with IL-4 had no effect on T-cell growth and differentiation. Therefore, it is likely that IL-4 effects T-cell development at an early stage corresponding with the *in vivo* data demonstrating a restricted temporal expression of IL-4 at an early point of fetal thymic development (Plum et al., 1990).

An *in vivo* study using transgenic mice that were engineered to selectively secrete IL-4 intrathymically, demonstrated similar results as the organ culture studies (Lewis et al., 1991). The DP cells from the transgenic mice had a significant increase in TCR expression relative to the control mice. Interestingly, these DP cells demonstrated a higher

activation response as measured by an increase in calcium concentration. In a model system for negative selection using cultured fetal thymus lobes, an increase in calcium mobilization follows stimulation of the DP cells via cross-linking the TCR that leads to the elimination of these cells (Finkel et al., 1989; Smith et al., 1989). Therefore, the DP cells from the transgenic mice may be more primed for negative selection than the normal mice, which may account for their reduced numbers (Lewis et al., 1991). Further work is necessary to determine if IL-4 is critical in negative and/or positive selection of developing thymocytes.

As mentioned above, the T-helper cells can be subdivided into distinct phenotypes based on their cytokine profile. The differential expression of certain cytokines by distinct Th subsets *ie.*Th1 and Th2, is likely to be important in the selection of the appropriate immunological response needed to effectively eliminate an invading pathogen.

In vitro studies with freshly isolated naive T-cells from mouse or human, demonstrated that IL-4 is a critical factor in the development of effector cells with the Th2 phenotype (Abehsira et al., 1992; Le-Gros et al., 1990; Maggi et al., 1992; Seder et al., 1992b; Swain et al., 1990).

The role for IL-4 in the selection of a Th2 response has also been demonstrated *in vivo* (Gross et al., 1993; Kopf et al., 1993; Le-Gros et al., 1990). There are several well studied mouse models which have demonstrated that the predominance of either a Th1 or Th2 response has a significant effect on the outcome of the disease (Sher et al., 1992). In one model, resistance to the leishmania parasite is dependent on T-cells that secret IFN-γ (Th1), while mice that are sensitive to parasite infection develop a Th2-like response marked by high levels of IL-4 production and low IFN-γ (Heinzel et al., 1989). Incubation with anti-IL-4 enables the

susceptible mice to resolve the disease (Chatelain et al., 1992; Sadick et al., 1991). In the anti-IL-4 treated mice a Th1 like response develops as indicated by an increase in IFN-γ expression. Recently, it was demonstrated that leishmania resistant mice engineered to overproduce IL-4 became susceptible to infection by the parasite and their serum IFN-γ levels were lower relative to the normal control mice (Ludmila et al., 1993).

The source of IL-4 *in vivo* has yet to be determined. In naive T-cells, there is little IL-4 production. It has been suggested that mast cells and/or basophils may provide the initial source of IL-4 during the antigenic priming process of T-cells (Paul et al., 1993; Romagnani, 1992). Also, it has not been determined if previously primed Th2 cells can maintain their phenotype *in vivo* without a constant source of IL-4. Therefore, it is possible that mast cells and/or basophils could play a role in sustaining the Th2 phenotype (Paul et al., 1993).

C. IL-4 Can Affect Non-Lymphoid Cells

Besides the up-regulation of $F_c\varepsilon RII$ on macrophages/monocytes, IL-4 has been shown to inhibit their production of proinflammatory mediators such as IL-1, TNF-α, and PGE_2 (Baumann and Gauldie, 1994). This suggests that IL-4 may act as an anti-inflammatory cytokine. Recently, it was shown that IL-4 increases production of the IL-1 receptor antagonist in human blood monocytes (Orino et al., 1992). Therefore, not only can IL-4 inhibit IL-1 expression by macrophages/monocytes, it can block the action of IL-1 through the receptor antagonist. IL-4 can also augment the surface expression of MHC class II on macrophages/monocytes (Paul, 1991).

Therefore, as with B-cells, IL-4 may regulate the antigen presentation function of macrophages.

As previously mentioned, IL-4 can act in concert with IL-3 as a growth factor for mast cells *in vitro*. On the other hand, some IL-3 independent transformed mast cell-lines that constitutively express IL-4 may depend on IL-4 as an autocrine growth factor. Incubation of a transformed mast cell line with anti-sense oligonucleotide to the IL-4 translation start site inhibited cell growth *in vitro* (M. Brown, unpublished data). Addition of antisense oligonucleotides to either the IL-2 start site or the sense strand of the IL-4 start site does not block growth. Interestingly, addition of anti-IL-4 to the cells does not block growth (Brown et al., 1987). Therefore, the autocrine growth of these cells may be mediated intracellularly by receptor-ligand interactions. There have been other tumor cells identified that demonstrate this type of autocrine interaction (Lang and Burgess, 1990). During an allergic response, mast cell numbers and it is possible that these cells may utilize IL-4 as an autocrine growth factor (extracellularly or intracellularly) to expand their numbers. Because IL-4 has been shown to be stored in mast cell granules, it is likely that the immediate release of this cytokine may have significant effects not only on mast cell growth but also activation potential (Bradding et al., 1992; Bradding et al., 1993). Recently, it was shown that pre-incubation of mouse CTMCs with IL-4, enhanced IgE mediated activation as measured by histamine release (Coleman et al., 1993b).

Cell adhesion molecules (CAM) play an important role in the migration of leukocytes to inflamed areas due to injury or infection. It has been shown that IL-4, either alone or synergistically with IL-1β, can upregulate vascular-CAM-1 (V-CAM-1) expression on microvascular

endothelium cells that in turn increases adhesiveness for lymphocytes (Masinovsky et al., 1990). Furthermore, it was demonstrated that IL-4 increases the adherence of T-cells to endothelial cells (Schleimer et al., 1992). IL-4 was also able to modulate the adherence of eosinophils and basophils, but not neutrophils, to V-CAM-1 on endothelial cells (Thornhill et al., 1990). Not only can IL-4 effect adhesion molecule expression on endothelial cells but regulates cytokine production by endothelial cells and act as a mitogen for capillary endothelial cells (Howells et al., 1991; Standiford et al., 1990; Toi et al., 1991).

IL-4 appears to play a role in hematopoiesis, although alone it does not appear to act as colony stimulating factor (CSF) (Paul, 1991). However, in combination with well defined CSFs, it can effect colony formation *in vitro*. In bone-marrow cultures, IL-4 enhances granulocytic colony formation in the presence of granulocyte-CSF and erythroid colony formation in the presence of erythropoietin (Broxmeyer et al., 1988; Peschel et al., 1989). IL-4 may also play a role in enhancing the viability of early hematopoietic progenitor cells. Incubation of IL-3 dependent bone-marrow derived progenitor cells or a multipotential progenitor with a calcium ionophore inhibited apoptosis (Roderiguez-Tarduchy et al., 1992). The soluble factor secreted by these cells that inhibited cell death was shown to be IL-4.

D. Antitumor Immunity

A tumor line stably transfected with an expression vector carrying the IL-4 cDNA was unable to form tumors in syngeneic mice and blocked tumor formation by a variety of other transplantable tumor lines (Tepper et al., 1989). This antitumor activity of IL-4 has been reported in another tumor model using renal carcinoma cells engineered to express IL-4 (Golumbek et al., 1991). The mechanism by which IL-4 mediated its antitumor effect is unclear. In the first study, the tumor site was infiltrated with macrophages and eosinophils (Tepper et al., 1989). IL-4 has been shown to increase the cytotoxic activity of macrophages to certain tumors (Paul, 1991). Further work demonstrated that the eosinophils were critical for the IL-4 mediated anti-tumor activity in this study (Tepper et al., 1992). It is unclear if IL-4 has a direct or indirect effect on eosinophil cytolytic activity. T-cell involvement was ruled out because the anti-tumor effect of IL-4 was seen in *nu/nu* mice (Tepper et al., 1992; Tepper et al., 1989). In the other reported case, macrophages, eosinophils and $CD8^+$ T-cells were found associated with the tumor (Golumbek et al., 1991). Again, IL-4 has been shown to modulate activity in all of these tumor infiltrating cell-types (Paul, 1991).

IL-4, like most other cytokines, plays a variety of roles in the developing immune/inflammatory response. Because of its pleiotropic effects at such low concentrations, its production must be tightly regulated. One level of regulation can occur at the point of gene transcription.

III Transcriptional Control of Gene Expression

The regulation of gene expression plays a key role in the development and function of cells within higher eukaryotes. Although there are many levels of control that can effect gene expression, the primary control point occurs at the level of transcription. Other mechanisms such as chromatin structure that mediates accessibility of a gene to transcription factors as well as mechanisms that help attenuate the level of product made, i.e. RNA splicing, mRNA stabilization, elongation, and translational control mechanisms, are also important in establishing a fine balance in the repertoire and level of genes expressed in eukaryotic cells. Because this thesis concentrates on transcriptional control of IL-4 expression, this overview will focus on the components of transcription and how they regulate differential gene expression.

A gene usually contains an array of cis-acting DNA elements located 5' and in some cases 3' of the +1 transcriptional start site. These DNA elements in turn bind both general protein factors at the initiation site and specific *trans*-acting proteins upstream and/or downstream of the start site. These *trans*-acting proteins serve as receivers of extracellular and intracellular signals that either initiate or block RNA synthesis in a temporally or tissue-specific manner. In eukaryotic organisms there are three types of RNA polymerases that mediate transcription: RNA Polymerase I, II and III. These polymerases initiate RNA synthesis from distinct types of genes: ribosomal genes, protein coding genes and 5S and tRNA genes, respectively. The focus of this section will be on transcriptional regulation of genes controlled by RNA Polymerase II.

A. *Cis*-acting DNA Elements

There are three types of *cis* elements: promoters, enhancers and negative regulatory elements (NRE). Both the promoter and enhancer regions are involved in the activation of transcription, while NREs down-regulate transcription.

1. Promoters

Promoters possess many regulatory elements or modules that control transcription of a gene (Nussinov, 1990; Wasylyk, 1988). The initiation module includes sequences from -45 to +30 that function in an orientation and position dependent fashion. This region binds several different general transcriptional factors that are important in establishing a preinitiation complex. These factors also help direct RNA polymerase II to the correct start site and are sufficient for low basal transcription (Nussinov, 1990; Wasylyk, 1988). This minimal promoter element usually contains an AT-rich segment with a TATAAA consensus sequence near -30 and a CA dinucleotide motif with the A located at the +1 start site. The TATAAA consensus sequence is similar to the Pribnow/Schaller "TATA-Box" found in prokaryotic promoters (Pribnow, 1975a; Schaller et al. 1975; Pribnow, 1975b). Systematic mutational analysis of either viral or mammalian promoters demonstrated that the TATA box is important for controlling the efficiency and the accuracy of transcription initiation (Benoist and Chambon, 1981; Concino et al., 1984; Corden et al., 1980; Grosschedl et al., 1981; Sassone et al., 1981). However, there have been several genes described which lack a TATA box. Studies on the SV40, terminal deoxynucleotidyltransferase (TdT) and dihydrofolate reductase (DHFR) genes that do not possess a TATA box, have identified sequence elements

called initiators that overlap the transcriptional start site and can direct low levels of transcription initiation (Ayer and Dynan, 1988; Means and Farnham, 1990; Smale and Baltimore, 1989). Initiator elements, however, can be found in promoters with a TATA box (Concino et al., 1984; Corden et al., 1980; Tokunaga et al., 1984). Recently, it was demonstrated that in the presence of a TATA box, the initiator element loses the ability to influence the direction and the location of transcription (O'Shea and Smale, 1992). Instead, it appears that the initiator in combination with the TATA box is important for modulating promoter strength, and that the TATA region retains control over the direction and site of transcription (O'Shea and Smale, 1992).

Additional promoter modules can be found upstream of the minimal promoter region. Typically, these elements are located between 45 and 200 bp upstream from the start site; however, in some cases promoter modules have been found downstream of the initiation site (Farnham and Means, 1990; Stenlund et al., 1987; Wasylyk, 1988). These sites are composed of short 10 to 20 bp sequences, bind one or more transcription factors and play an important role in augmenting the transcription rate from the basal promoter in an orientation independent fashion (Wasylyk, 1988). Certain elements such as the GGGGCGG box or the CCAAT box bind ubiquitous factors and are found in a large number of promoters (Wasylyk, 1988). Other proximal promoter elements in combination with the ubiquitous factors, play an important role in regulating tissue-specific promoter activity in response to extracellular cues or during different stages of development (Dedrick and Jones, 1990; Evans et al., 1990; Maire et al., 1989; Serfling et al., 1989).

There are also examples of genes with more than one initiation site that are independently controlled by different proximal promoter elements (Schibler and Sierra, 1987). The use of alternative promoters can provide another form of regulation to control the temporal and/or tissue-specific expression of a gene (Schibler and Sierra, 1987). Also, alternative promoters elements can yield different transcripts of various lengths that may be translated into different isomers (Kozak, 1988). Alternatively transcribed isomers may possess antagonistic function *eg.* the long isoform of the E2 transcription factor from bovine papilloma virus acts as an activator while the short form acts as a repressor (Lambert et al., 1987). Using alternative promoters may yield two proteins in which one form is localized to the cytoplasm and the other is secreted *eg.* yeast invertase SUC2 (Carlson et al., 1983). Finally, one promoter may attenuate the activity of another. The c-myc gene has four upstream promoters (P0, P1, P2 and P3) and a 3' promoter that transcribes antisense RNA which may play a role in modulating the level of sense RNA (Marcu et al., 1992; Spicer and Sonenshein, 1992). In normal cells, 80-90% of steady-state c-Myc RNA is due to the P2 promoter (Taub et al., 1984). The ratio of P2 to P1 promoter activity ranges from 5 to above 10. Most transcripts from the upstream P1 promoter of the mouse c-Myc gene were shown to be prematurely terminated at the P2 promoter (Wright et al., 1991). It is possible that early termination of P1 may select for transcription from the P2 promoter (Wright et al., 1991). Further work has shown that transcriptional activity of c-myc is regulated by the rate of release of RNA polymerase II from the P2 promoter (Strobl and Eick, 1992). In several malignant cell-lines (*eg.* Burkitts lymphoma cells (BL)), there is an increase in c-myc expression due to translocation events that in many cases

leads to an increase in P1 promoter usage (Marcu et al., 1992). It was shown in one study that, in BL cells, pausing at the P2 promoter was diminished (Strobl et al., 1993). The rate of initiation from both P1 and P2 was the same in both normal and BL cells. Therefore, the P2 promoter region appears to attenuate P1 promoter activity by blocking elongation.

2. Enhancer Elements

The term "enhancer" was coined after it was observed that a 72 bp repeat from the SV40 virus could augment transcription in either orientation and from a considerable distance upstream or downstream from the SV40 promoter or a heterologous promoter (Moreau et al., 1981). Subsequently, several cellular enhancers have been described which can also function in an orientation and position independent fashion up to several kilobases upstream or downstream from the promoter (Gillies et al., 1983; Goodbourn et al., 1985; Ho et al., 1989; Vogel et al., 1986). Similar to promoters, enhancers are multimodule elements each of which binds one or more transcription factors (Wasylyk, 1988). In many cases the same binding sequences can be found in both promoters and enhancers (Wasylyk, 1988). It is believed that enhancer elements and proximal promoter elements function similarly and that their distinction is based more on their location to the minimal promoter (Wasylyk, 1988). Therefore, enhancers can be considered as distal promoter elements. This ability for enhancers to increase promoter activity at a distance may be a consequence of the arrangement and number of modules they contain (Maniatis et al., 1987). The most well studied prototypic enhancer is the 72 bp SV40 enhancer. Extensive mutational/functional analysis identified several layers of organization within this enhancer element. The enhancer

was shown to contain three distinct modules that co-operate with one another or with duplicates of themselves to regulate enhancer activity (Herr and Clarke, 1986). The modules could be further subdivided into short sequences called enhansons, that serve as protein binding sites (Ondek et al., 1988). Individual enhansons could be duplicated or interchanged to form new enhancer elements (Ondek et al., 1988). Based on the SV40 enhancer model, an enhancer can be composed of several modules, with each module containing either one enhanson, two repeated enhansons or two different combinations of enhansons (Dynan, 1989). The contribution of a particular enhanson to enhancer function may vary from cell to cell reflecting the relative abundance and/or activity of specific transcription factors (Dynan, 1989). Examples of this type of organization in cellular enhancers can be found in the immunoglobulin heavy and light chain enhancer and the α T-cell receptor enhancer (Ho et al., 1989; Lenardo et al., 1987).

In addition to DNA-protein interactions, transcription depends on direct protein-protein contact of the specific regulatory protein with the general components of the preinitiation complex at the promoter start site. Therefore, it is unclear how an enhancer element, located in many cases thousands of base pairs away from the initiation site can augment transcription. There have been three models proposed to explain enhancer activity at a distance (Echols, 1990): the first model or scanning model proposes that the regulatory protein binds to the distal site and traverses the DNA toward the initiation site in order to augment transcription; the second or structural transmission model proposes that the binding of the enhancer protein facilitates a conformational change in the DNA structure that is propagated from the enhancer to the promoter allowing other

proteins to bind at the initiation site and begin transcription; the third model suggests that the enhancer bound proteins come in direct contact with the promoter region by the looping out of the intervening sequence (looping model) and thereby increase promoter activity. The looping model has gained the most favor as the mechanism for bringing distal bound factors in contact with the initiation site. This is based, in part, on several studies in both prokaryotic and eukaryotic systems that have visualized DNA looping interactions of transcription regulators bound at separate sites using electron microscopy (Griffith et al., 1986; Kramer et al., 1987; Su et al., 1991).

3. Negative Regulatory Sequences

Besides containing positive regulating *cis* elements, many genes contain negative regulatory elements (NRE) (Jackson, 1991; Renkawitz, 1990). Negative elements may keep a gene inactive in tissues where the gene should be silent. The Ig κ gene contains an NRE adjacent to the intronic enhancer that is important for silencing the kappa gene in non-B cells (Pierce et al., 1991). Alternatively, negative elements may keep a gene silenced until an appropriate signal is received to turn the gene on (Renkawitz, 1990). Once the stimulus is removed, then the same or other negative elements are needed to shut-off the gene. An example of this type of negative regulation occurs in the interferon β promoter. The interferon-β promoter contains an NRE that blocks the constitutive activity of NF-κB (Nourbakhsh et al., 1993). Upon virus induction the NRE activity is removed.

In some regulatory regions of a gene the binding sequence for positive and negative protein factors overlap and therefore, a direct

competition occurs for that site. An example of this type of competition is illustrated in the regulation of the SSA1 gene encoding HSP70 in *S. cerevisiae* where both the positive heat shock factor and the upstream repressor factor compete for their partially overlapping cognate sites (Park and Craig, 1989). In other situations the binding site can be the same for both positive and negative factors as in the case for CREB and CREM, respectively (Foulkes et al., 1991). CREM homodimers either compete directly for binding with CREB homodimers or a CREM/CREB heterodimer is formed that can bind the cAMP response element (CRE) but is a weak trans-activator (Meyer and Habener, 1993). Some negative *cis* elements do not have to overlap the positive element but can be found adjacent to them. These NRE sites are position dependent. Positive factors can bind to their sites but cannot transmit their signal to the transcription initiation complex due to interference from the repressor factors bound to adjacent NREs (Jackson, 1991; Renkawitz, 1990). Examples of this type of system include the c-myc gene (the negative factor myc-PRF binds next to myc-CF1) and the α-fetoprotein gene (Kakkis et al., 1989; Nakabayashi et al., 1991). Finally, another type of negative regulating sequence has been identified that can function in an orientation and position independent fashion, similar to enhancers (Jackson, 1991). This type of element is called a silencer and like an enhancer can have many different modules. Examples of genes that contain silencers include the glutathione transferase P gene, the type IV collengenase gene and the immunoglobulin kappa gene (Frisch and Morisaki, 1990; Imagawa et al., 1991; Pierce et al., 1991). A proposed mechanism for silencers is that they directly repress initiation (Renkawitz, 1990).

B. *Trans*-acting Factors

The multiple combinations of positive and negative *cis*-acting elements arrayed in unique configurations confer on each gene an individualized spatial and temporal transcription program (Mitchell and Tjian, 1989). The function of the *cis*-acting DNA element, however, is dependent on the factor or factors that bind to it.

1. General Structural Features

The factors that bind to *cis*-acting DNA elements can be detected in nuclear extract preparations using various *in vitro* DNA binding assays (Mitchell and Tjian, 1989). In turn many of these factors have been purified and cloned. Structure-function studies have shown that different DNA binding proteins are composed of a variety of separable DNA binding and activation domains (Mitchell and Tjian, 1989).

The DNA binding domains of several mammalian factors have been typically localized to a small subregion of 60 to 100 amino acids (Mitchell and Tjian, 1989). There are different types of transcription factors that have distinct structural motifs necessary for DNA binding (Krajewska, 1992; Pabo and Sauer, 1992): zinc-finger, helix-turn-helix (HTH) or homeodomain, helix-loop-helix (HLH) and leucine zipper proteins (b-ZIP). Transcription factors such as SP-1, GATA and steroid hormone receptors contain the zinc finger binding motif. The zinc finger consists of ~30 amino acid residues, looped in a finger-like structure. Two cysteine and two histidine residues separated by ~12 amino acids, stabilize the finger domain by tetrahedrally coordinating with Zn^{+2} (Mitchell and Tjian, 1989). The steroid hormone receptors *eg.* glucocorticoid receptor have two pairs of cyteines that coordinate Zn^{+2} (Mitchell and Tjian, 1989). The

HTH motif is distantly related to the helix-turn-helix motif described for some prokaryotic repressor proteins, e.g. cro (Pabo and Sauer, 1992). This motif is characterized by three alpha helical DNA binding domains joined by amino acids that form a turn in the structure (Brennan and Matthews, 1989). One class of eukaryotic DNA binding proteins that contain this motif are the homeodomain transcription factors *eg.* Oct 1, *Drosophila* antennapedia and engrailed (Pabo and Sauer, 1992). The HLH binding domain proteins *eg.* CTF/NF-I and MyoD, bind to DNA as a dimer (Krajewska, 1992). The HLH proteins are composed of several basic amino acids necessary for DNA recognition (Krajewska, 1992). In addition, a helix-loop-helix structure is formed with two amphipathic α helices about 15 amino acids in length with a non-defined loop of 9-20 amino acids separating them. This HLH structure is important for dimerization (Krajewska, 1992). A fourth type of transcriptional binding protein also binds as a dimer and is characterized by a highly basic DNA binding domain and several heptad repeats with leucine amino acids at every fourth residue within the heptad (Pabo and Sauer, 1992). The latter segment is referred to as the "leucine zipper" because the heptad repeats form an amphipathic helix with the leucine residues on one side of the helix (Mitchell and Tjian, 1989). The leucine zipper configuration is important for dimerization between the monomers; dimerization in turn is necessary for DNA binding (Mitchell and Tjian, 1989). The family of transcription factors that have this motif are referred to as "b-ZIP". Examples of b-ZIP proteins include CREB, ATF and AP-1 (Mitchell and Tjian, 1989). Some factors, such as c-myc have a combination of both an HLH and a b-ZIP binding motif (Prendergast, 1989). There are also, other DNA binding factors whose binding domains are unclassified *eg.* the serum

response factor and ets factors (Karim et al., 1990; Norman et al., 1988). The DNA binding factors recognize short sequences (*eg.* GGGGCGG (SP1) or TGACTCA (AP-1)) which they bind as either monomers or as dimers (Mitchell and Tjian, 1989). A majority of the transcription factors have undergone evolutionary changes leading to a family of factors that can recognize the same DNA binding site. For example, members of the AP-1 b-ZIP family include c-jun, junB, junD, c-fos, fra-1, fra-2 and fosB (Meyer and Habener, 1993). The AP-1 complex is typically comprised of jun/fos heterodimer combinations, but jun/jun homodimers can also bind the AP-1 site (Turner and Tjian, 1989).

The function of the DNA binding domain is to bring the activating surface of the protein to the vicinity of a gene. The activation domains are believed to facilitate the formation of the preinitiation complex through protein-protein interactions with the components of the general transcription machinery (Ptashne, 1988). The activation domains are also composed of short regions (30-100) of amino acid residues (Mitchell and Tjian, 1989). There are three classes of activation domains: acidic activators characterized by a high proportion of acidic amino acid residues found on one side of an α helix (Ptashne, 1988), glutamine-rich domains (Courey et al., 1989) and proline rich activators (Mermod et al., 1989). Examples of transcription factors with these types of domains include the yeast factor Gal4 and the mammalian factors SP1 and CTF, respectively (Mitchell and Tjian, 1989).

2. Mode of Action

The activation domains as mentioned above, are believed to play a role in augmenting preinitiation complex (pic) assembly. The initiation

complex is composed of many highly conserved general factors: TFIIA-, B,- D,- E,- F,-G and RNA polymerase II (Conaway and Conaway, 1993). The TFIID complex is believed to be the key component of this complex formation and acts as the link between the activator proteins and the general transcription machinery (Pugh and Tjian, 1992). TFIID is composed of several subunits: a TATA-binding protein (TBP) that binds to the TATA box, and several TBP associated factors or TAFs (Conaway and Conaway, 1993). TFIID is the first general factor to bind to the promoter during pic assembly. Early work demonstrated that TFIID or just TBP binding could displace nucleosome formation at the promoter region and relieve nucleosome repression of transcription initiation (Meisterernst et al., 1990). It has been shown that various activator proteins, such as the Gal4-VP16 chimera (the yeast Gal4 DNA binding domain fused to the herpes simplex VP16 activation domain), SP1 and USF, could enhance the ability of TFIID complex or recombinant yeast TBP to block nucleosome formation at the promoter and therefore, enhance transcription initiation (Croston et al., 1991; Workman et al., 1991). This correlated with earlier work demonstrating that activator proteins could enhance the interaction of TFIID with the promoter (Horikoshi et al., 1988a; Horikoshi et al., 1988b). Some activator factors such as VP16 appear to interact directly with the transcriptional machinery (Lin et al., 1991; Stringer et al., 1990). However, this does not explain how synergistic actions occur with multiple bound activators or between such diverse types of activating proteins. It has been suggested that there may be other adapter or coactivator molecules that are necessary for mediating protein-protein interactions between multiple and/or different combinations of activating factors with the general transcription machinery at the initiation site. Various studies

have provided evidence for such mediator proteins (Berger et al., 1990; Carey et al., 1990; Flanagan et al., 1991; Kelleher et al., 1990; Pugh and Tjian, 1990; Tanese et al., 1991). Because of the recent discovery that TAFs are associated with the TFIID general transcription complex, it has been postulated that these factors may act as adapter or co-activator molecules (Pugh and Tjian, 1992). Recently, one of the TAFs (TAF 110) was purified from Drosophila and shown to interact directly with the transcription factor SP1 (Hoey et al., 1993). It has been further postulated that there may be different TFIID complexes with different subsets of TAFs at various promoters (Pugh and Tjian, 1992). The distinct array of *cis* elements bound by transcription factors may determine which TFIID complex associates with the promoter initiation site (Pugh and Tjian, 1992).

3. Regulation of Activity and Diversity

Although much of the diversity in multicellular organisms depends simply upon the restricted expression of transcription factors in the various cell types, variations in the activities of the proteins can also make a major contribution (Struhl, 1991). Differences in protein activity can occur at the level of DNA binding, inherent transcriptional activation potential, or protein-protein interactions (Struhl, 1991).

Sequestering of factors in the cytoplasm until needed is one form of regulating transcription factor function that is unique to eukaryotes. Two examples of this type of regulation are seen with both the NF-κB and NF-AT transcription factors. The p65 component of NF-κB is sequestered in the cytoplasm by the association with I-κB inhibitory protein (Baeuerle and Baltimore, 1988a). Based on *in vitro* studies, the IκB inhibitory

component is phosphorylated upon mitogenic activation, releasing the p65 NFκB subunit and allowing it to travel to the nucleus where it dimerizes with the p50 subunit to activate transcription (Baeuerle and Baltimore, 1988b). The T-cell factor NF-AT, on the other hand, appears to be retained in the cytoplasm without an additional factor. A model based on various biochemical and molecular studies, proposes that upon activation of the T-cell, NF-AT is dephosphorylated by the calmodulin-dependent phosphatase calcineurin resulting in the translocation of NF-AT to the nucleus (Rao, 1994; Schreiber and Crabtree, 1992).

Probably the main form of postranslational modification that affects the function of the various *trans*-acting factors is mediated by phosphorylation or dephosphorylation of the proteins (Hunter and Karin, 1992). As shown above, the regulation of nuclear translocation is mediated through different states of phosphorylation. The DNA binding function of some transcription factors can also be modulated through variations in phosphorylation states. The c-Jun binding domain is constitutively phosphorylated in resting epithelial and fibroblast cells (Boyle et al., 1991). In this state, c-Jun cannot bind efficiently to its cognate site. Upon stimulation of these cells with a phorbol ester, however, c-Jun is partially dephosphorylated and can bind with high affinity to the AP-1 site (Boyle et al., 1991). Some proteins can bind whether they are phosphorylated or not. Therefore, another role for phosphorylation is in the regulation of the activation domain. In most cases, phosphorylation of the activation domain leads to positive effects on transactivation. CREB is a member of the b-Zip family and binds to the palindrome sequence TGACGTCA (CRE) as a dimer (Meyer and Habener, 1993). It is activated through the cAMP dependent pathway via protein

kinase A (PKA). PKA phosphorylation does not appear to play a role in DNA binding or dimerization *in vitro*, however, it appears to be important in the binding of CREB to asymmetrical CREs (Nichols et al., 1992; Yamamoto et al., 1988). On the other hand, PKA phosphorylation of ser-133 (CREB-343 isoform) or ser-119 (CREB-327 isoform) is important to the activity of this factor (Meyer and Habener, 1993). These residues are outside the DNA binding domain and lie within the glutamine-rich activation domain (Meyer and Habener, 1993). Although PKA phosphorylation is essential for activation it is not sufficient. A sequence located carboxy-proximal to ser 133 was shown to be required for transactivation (Gonzalez et al., 1991). It is possible that phosphorylation alters the conformation of CREB to expose the critical sequence(s) for transactivation (Gonzalez et al., 1991).

The yeast ADR1 factor is an example of negative or down-regulation of transactivation due to phosphorylation. Repression by ADR1 requires PKA phosphorylation (Cherry et al., 1989). ADR1 DNA binding appears to be independent of phosphorylation. Therefore, it is likely that phosphorylation inhibits the interaction of the ADR1 protein with the general transcriptional machinery (Taylor and Young, 1990).

Another form of regulation that has been described for the AP-1 components, Fos and Jun, involves reduction-oxidation (redox) that increases the binding activity of AP-1 (Xanthoudakis et al., 1992). The factor mediating this redox effect was isolated and shown to be a DNA repair enzyme (Xanthoudakis and Curran, 1992). This same DNA repair enzyme also increased the binding affinity of NFkB, Myb and CREB/ATF1 family members (Xanthoudakis and Curran, 1992).

Heterodimerization between members of the same family of protein factors that recognize the same cognate binding site can also regulate function. Heteromers can result in different types of factors relative to the homodimer parent, in terms of their DNA binding and/or activation capabilities (Struhl, 1991). For example, it was recently shown that different heteromers within the NF-κB *rel* family differentially bind to κB sites from either the IL-2 promoter or the HIV LTR (Perkins et al., 1992). Also, within this same experiment, there was differences in activation capabilities among the various heteromers that was independent of binding (Perkins et al., 1992). Dimerization between members of different families can also occur. This was demonstrated through cross-dimerization of b-ZIP proteins from the AP-1 family with members of the ATF1/CREB family (Hai and Curran, 1991). In this study, cross family dimerization altered the DNA binding specificity relative to their parent homodimers (Hai and Curran, 1991). Cross-family dimerization may also create different combinations of activation domains with unique requirements for *trans*-activation. The ability to cross-dimerize can therefore, expand the repertoire of transcriptional factors and provide another level of control over differential gene expression.

Finally, protein-protein interactions between transcription factors can have different effects on regulating transcription. First, a DNA binding protein may have low activation capabilities that can be circumvented by interacting with a non-DNA binding protein with a strong activation domain. An example of this is seen with the interaction of the herpes simplex VP16 protein and the inactive DNA bound Oct1 transcription factor (Stern et al., 1989). A more recent example is seen with the NF-AT complex that regulates IL-2 gene expression. The

cytoplasmic (NF-AT_p) component of NF-AT was recently purified (McCaffrey et al., 1993). This factor can bind the IL-2 NF-AT binding site but is unable to activate transcription. Earlier work had shown that the nuclear NF-AT complex consists of NF-AT_p and AP-1 (Jain et al., 1992). Co-addition of Jun or Jun/Fos with NF-AT_p resulted in a significant increase in transcriptional activation (McCaffrey et al., 1993). Protein-protein interactions can affect cooperative binding either between similar proteins as in the case for steroid receptor hormones or between two distinct proteins, like the yeast factors MCM1 and α2 (Keleher et al., 1988; Keleher et al., 1989; Tsai et al., 1989). Finally, protein-protein interactions between two DNA binding factors can either augment or inhibit transcription as in the case of NFκB/serum response factor or glucocorticoid receptor/c-Jun interactions, respectively (Kuang et al., 1993; Yang-Yen et al., 1990). In the end, the abundance and/or the ability of these various transcriptional factors to function in different cell types, under various physiological conditions and in different combinations will help to determine when and how much a gene will be expressed.

References

Abe, E., R.D. Malefyt, I. Matsuda, K. Arai and N. Arai. (1992), "An 11 bp DNA sequence motif apparently unique to the human IL-4 gene confers responsiveness to T cell activation signals." Proc. Natl. Acad. Sci. USA, **89**: 2864-2868.

Abe, T., H. Ochiai, Y. Minamishima and Y. Nawa. (1988), "Induction of intestinal mastocytosis in nude mice by repeated injection of interleukin-3." International Archives of Allergy & Applied Immunology, **86**: 356-358.

Abehsira-Amar O., M. Gibert, M. Joliy, J. Theze and D. L. Jankovic. (1992), "IL-4 plays a dominant role in the differential development of Th0 into Th1 and Th2 cells." Journal of Immunology, **148**: 3820-3829.

Alam, R., P. A. Forsythe, S. Stafford, Brown Ma Lett and J. A. Grant. (1992), "Macrophage inflammatory protein-1 alpha activates basophils and mast cells." Journal of Experimental Medicine, **176**: 781-786.

Anderson, D.M., S.D. Lyman, A. Baird, J.M. Wignall, J. Eisenman, C. Rauch, C.J. March, H.S. Boswell, S.D. Gimpel, D. Cosman and D.E. Williams. (1990), "Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms." Cell, **63**: 235-243.

Ayer, D. E. and W. S. Dynan. (1988), "Simian virus 40 major late promoter: a novel tripartite structure that includes intragenic sequences." Molecular & Cellular Biology, **8**: 2021-2033.

Baeuerle, P. A. and D. Baltimore. (1988a), "Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor." Cell, **53**: 211-217.

Baeuerle, P. A. and D. Baltimore. (1988b), "I kappa B: a specific inhibitor of the NF-kappa B transcription factor." Science, **242**: 540-6.

Baumann, H and J Gauldie. (1994), "The acute phase response." Immunology Today, **15**: 74-80.

Ben-Sasson, S., G. Le Gros, D.H. Conrad, F.D. Finkelman and W.E. Paul. (1990), "Cross-linking Fc receptors stimulate splenic non-B, non-T cells to

secrete interleukin 4 and other lymphokines." Proc. Natl. Acad. Sci USA, **87**: 1421-1425.

Benoist, C. and P. Chambon. (1981), "In vivo sequence requirements of the SV40 early promotor region." Nature, **290**: 304-310.

Berger, S. L., W. D. Cress, A. Cress, S. J. Triezenberg and L. Guarente. (1990), "Selective inhibition of activated but not basal transcription by the acidic activation domain of VP16: evidence for transcriptional adaptors." Cell, **61**: 1199-208.

Borish, L. and B. Z. Joseph. (1992), "Inflammation and the allergic response. [Review]." Medical Clinics of North America, **76**: 765-87.

Boyle, W.J., T. Smeal, L.H. Defize, P. Angel, J.R. Woodgett, M. Karin and T. Hunter. (1991), "Activation of protein kinase C decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity." Cell, **64**: 573-584.

Bradding, P., I.H. Feather, P.H. Howarth, R. Mueller, J.A. Roberts, K. Britten, J.P.A. Bews, T.C. Hunt, Y. Okayama, C.H. Heusser, G.R. Bullock, M.K. Church and S.T. Holgate. (1992), "Interleukin 4 is localized to and released by human mast cells." J. Exp. Med., **176**: 1381-1386.

Bradding, P., I.H. Feather, S. Wilson, P.G. Bardin, C.H. Heusser, S.T. Holgate and P.H. Howarth. (1993), "Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects." J Immunol, **151**: 3853-3865.

Brennan, R. G. and B. W. Matthews. (1989), "The helix-turn-helix DNA binding motif. [Review]." Journal of Biological Chemistry, **264**: 1903-1906.

Brown, M.A., J.H. Pierce, C.J. Watson, J. Falco, J.N. Ihle and W.E. Paul. (1987), "B cell stimulatroy factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells." Cell, **50**: 809-818.

Broxmeyer, H. E., L. Lu, S. Cooper, R. Tushinski, D. Mochizuki, B. Y. Rubin, S. Gillis and D. E. Williams. (1988), "Synergistic effects of purified recombinant human and murine B cell growth factor-1/IL-4 on colony formation in vitro by hematopoietic progenitor cells. Multiple actions." Journal of Immunology, **141**: 3852-3862.

Bruhn, K.W., K. Nelms, J. Boulay, W.E. Paul and M. J. Lenardo. (1993), "Molecular dissection of the mouse interleukin-4 promoter." Proc. Natl. Acad. Sci. USA, **90**: 9707-9711.

Burd, P.R., H.W. Rogers, J.R. Gordon, C.A. Martin, J. Sundararajan, S.D. Wilson, A.M. Dvorak, S.J. Galli and M.E. Dorf. (1989), "Interleukin 3-dependent and -independent mast cells stimulated with IgE and antigen express multiple cytokines." J. Exp. Med., **170**: 245-257.

Capron, A., J.P. Dessaint, M. Capron, M. Joseph, J.C. Ameisen and A.B. Tonnel. (1986), "From parasites to allergy: a second receptor for IgE." Immunology Today, **7**: 15-18.

Carding, S. R., E. J. Jenkinson, R. Kingston, A. C. Hayday, K. Bottomly and J. J. Owen. (1989), "Developmental control of lymphokine gene expression in fetal thymocytes during T-cell ontogeny." Proc Natl Acad Sci U S A, **86**: 3342-3345.

Carey, M., Y. S. Lin, M. R. Green and M. Ptashne. (1990), "A mechanism for synergistic activation of a mammalian gene by GAL4 derivatives." Nature, **345**: 361-364.

Carlson, M., R. Taussig, S. Kustu and D. Botstein. (1983), "The secreted form of invertase in *S. cerevisiae* is synthesized from mRNA encoding a signal sequence." Mol. Cell. Biol., **3**: 439-447.

Chatelain, R., K. Varkila and R.L. Coffman. (1992), "IL-4 induces a Th2 response in *Leishmania major* infected mice." J Immunol, **148**: 1182-1187.

Cherry, J. R., T. R. Johnson, C. Dollard, J. R. Shuster and C. L. Denis. (1989), "Cyclic AMP-dependent protein kinase phosphorylates and inactivates the yeast transcriptional activator ADR1." Cell, **56**: 409-419.

Chung, S. W., P. M. Wong, Ong G. Shen, S. Ruscetti, T. Ishizaka and C. J. Eaves. (1986), "Production of granulocyte-macrophage colony-stimulating factor by Abelson virus-induced tumorigenic mast cell lines." Blood, **68**: 1074-1081.

Coffman, R.L., J. Ohara, M.W. Bond, J. Carty, A. Zlotnick and W.E. Paul. (1986), "B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells." J. Immunol., **136**: 4538-4541.

Coleman, JW, MR Holliday, I Kimber, KM Zsebo and SJ Galli. (1993a), "Regulation of mouse peritoneal mast cell secretory function by stem cell factor, IL-3 or IL-4." J. Immunol, **159**: 556-562.

Coleman, J. W., M. R. Holliday, I. Kimber, K. M. Zsebo and S. J. Galli. (1993b), "Regulation of mouse peritoneal mast cell secretory function by stem cell factor, IL-3 or IL-4." Journal of Immunology, **150**: 556-562.

Conaway, R. C. and J. W. Conaway. (1993), "General initiation factors for RNA polymerase II. [Review]." Annual Review of Biochemistry, **62**: 161-190.

Concino, M. F., R. F. Lee, J. P. Merryweather and R. Weinmann. (1984), "The adenovirus major late promoter TATA box and initiation site are both necessary for transcription in vitro." Nucleic Acids Research, **12**: 7423-7433.

Corden, J., B. Wasylyk, A. Buchwalder, Corsi P. Sassone, C. Kedinger and P. Chambon. (1980), "Promoter sequences of eukaryotic protein-coding genes." Science, **209**: 1406-1414.

Courey, A. J., D. A. Holtzman, S. P. Jackson and R. Tjian. (1989), "Synergistic activation by the glutamine-rich domains of human transcription factor Sp1." Cell, **59**: 827-836.

Croston, G. E., L. A. Kerrigan, L. M. Lira, D. R. Marshak and J. T. Kadonaga. (1991), "Sequence-specific antirepression of histone H1-mediated inhibition of basal RNA polymerase II transcription." Science, **251**: 643-649.

Dayton, E. T., P. Pharr, M. Ogawa, W. E. Serafin, K. F. Austen, Schaffer F. Levi and R. L. Stevens. (1988), "3T3 fibroblasts induce cloned interleukin 3-dependent mouse mast cells to resemble connective tissue mast cells in granular constituency." Proceedings of the National Academy of Sciences of the United States of America, **85**: 569-572.

Dedrick, R. L. and P. P. Jones. (1990), "Sequence elements required for activity of a murine major histocompatibility complex class II promoter bind common and cell-type-specific nuclear factors." Molecular & Cellular Biology, **10**: 593-604.

Del Prete, G., E. Maggi, P. Parronchi, I. Chretien, A. Tiri, D. Macchia, M. Ricci, J. Banchereau, Vries J. De and S. Romagnani. (1988), "IL-4 is an

essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants." Journal of Immunology, **140**: 4193-8.

Delespesse, G., M. Sarfati, C. Y. Wu, S. Fournier and M. Letellier. (1992), "The low-affinity receptor for IgE. [Review]." Immunological Reviews, **125**: 77-97.

Denburg, J. A. (1992), "Basophil and mast cell lineages in vitro and in vivo [published erratum appears in Blood 1992 Aug 1;80(3):855]. [Review]." Blood, **79**: 846-860.

Dynan, W. S. (1989), "Modularity in promoters and enhancers. [Review]." Cell, **58**: 1-4.

Echols, H. (1990), "Nucleoprotein structures initiating DNA replication, transcription, and site-specific recombination. [Review]." Journal of Biological Chemistry, **265**: 14697-14700.

Enerback, L. and K. Norrby. (1989), "The Mast Cells." Current Topics in Pathology, **79**: 169-204.

Evans, T., G. Felsenfeld and M. Reitman. (1990), "Control of globin gene transcription. [Review]." Annual Review of Cell Biology, **6**: 95-124.

Farnham, P. J. and A. L. Means. (1990), "Sequences downstream of the transcription initiation site modulate the activity of the murine dihydrofolate reductase promoter." Molecular and Cellular Biology, **10**: 1390-1398.

Fernandez, Botran R., V. M. Sanders, T. R. Mosmann and E. S. Vitetta. (1988), "Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells." Journal of Experimental Medicine, **168**: 543-558.

Finkel, T. H., J. C. Cambier, R. T. Kubo, W. K. Born, P. Marrack and J. W. Kappler. (1989), "The thymus has two functionally distinct populations of immature alpha beta + T cells: one population is deleted by ligation of alpha beta TCR." Cell, **58**: 1047-1054.

Finkelman, F. D., J. Holmes, I. M. Katona, Jf Jr Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann and W. E. Paul. (1990), "Lymphokine control of in vivo immunoglobulin isotype selection. [Review]." Annual Review of Immunology, **8**: 303-333.

Finkelman, F. D., I. M. Katona, Jf Jr Urban, C. M. Snapper, J. Ohara and W. E. Paul. (1986), "Suppression of in vivo polyclonal IgE responses by monoclonal antibody to the lymphokine B-cell stimulatory factor 1." Proceedings of the National Academy of Sciences of the United States of America, **83**: 9675-9678.

Firestein, G. S., W. D. Roeder, J. A. Laxer, K. S. Townsend, C. T. Weaver, J. T. Hom, J. Linton, B. E. Torbett and A. L. Glasebrook. (1989), "A new murine CD4+ T cell subset with an unrestricted cytokine profile." Journal of Immunology, **143**: 518-525.

Flanagan, P. M., R.J. Kelleher, M. H. Sayre, H. Tschochner and R. D. Kornberg. (1991), "A mediator required for activation of RNA polymerase II transcription in vitro." Nature, **350**: 436-438.

Foulkes, N. S., E. Borrelli and C. P. Sassone. (1991), "CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription." Cell, **64**: 739-749.

Frisch, S. M. and J. H. Morisaki. (1990), "Positive and negative transcriptional elements of the human type IV collagenase gene." Molecular & Cellular Biology, **10**: 6524-6532.

Galli, SJ. (1987), "New approaches for the analysis of mast cell maturation , heterogeneity, and function." FASEB, **46**: 1906-1913.

Galli, SJ and LM Lichtenstein. 1988. Biology of mast cells and basophils. third ed. Edited by E. J. Middleton, C. Reed, E. Ellis, N. J. Adkinson and J. Yunginger. Allergy: Principles and Practice 106-134.

Galli, S.J. (1990), "Biology of Disease New insights into "the riddle of the mast cells:" Microenvironmental regulation of mast cell development and phenotypic heterogeneity." Lab. Invest., **62**: 5-33.

Galli, S.J. (1993), "New Concepts about the Mast cell." N. Eng. J. of Med., **328**: 257-265.

Geissler, E. N., M. A. Ryan and D. E. Housman. (1988), "The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene." Cell, **55**: 185-192.

Gillies, S. D., S. L. Morrison, V. T. Oi and S. Tonegawa. (1983), "A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene." Cell, **33**: 717-728.

Golumbek, P.T., A.J. Lazenby, H.I. Levitsky, L.M. Jaffee, H. Karasuyama, M. Baker and D.M. Pardoll. (1991), "Treatment of established renal cancer by tumor cells engineered to secrete interleukin-4." Science, **254**: 713-716.

Gonzalez, G. A., P. Menzel, J. Leonard, W. H. Fischer and M. R. Montminy. (1991), "Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB." Molecular & Cellular Biology, **11**: 1306-1312.

Goodbourn, S., K. Zinn and T. Maniatis. (1985), "Human beta-interferon gene expression is regulated by an inducible enhancer element." Cell, **41**: 509-520.

Gordon, J.R., P.R. Burd and S.J. Galli. (1990), "Mast cells as a source of multifunctional cytokines." Imm. Today, **11**: 458-464.

Gordon, J.R. and S.J. Galli. (1991), "Release of both preformed and newly synthesized tumor necrosis factor α (TNF-α)/cachectin by mouse mast cells stimulated via the FcεRI. A mechanism for the sustained action of mast cell-derived TNF-α during IgE-dependent biological responses." J. Exp. Med., **174**: 103-107.

Greenbaum, L. A., J. B. Horowitz, A. Woods, T. Pasqualini, E. P. Reich and K. Bottomly. (1988), "Autocrine growth of CD4+ T cells. Differential effects of IL-1 on helper and inflammatory T cells." Journal of Immunology, **140**: 1555-1560.

Griffith, J., A. Hochschild and M. Ptashne. (1986), "DNA loops induced by cooperative binding of lambda repressor." Nature, **322**: 750-752.

Gross, A., S. Z. Ben-Sasson and W. E. Paul. (1993), "Anti-IL-4 diminishes in vivo priming for antigen-specific IL-4 production by T cells." Journal of Immunology, **150**: 2112-2120.

Grosschedl, R., B. Wasylyk, P. Chambon and M. L. Birnstiel. (1981), "Point mutation in the TATA box curtails expression of sea urchin H2A histone gene in vivo." Nature, **294**: 178-180.

Hai, T. and T. Curran. (1991), "Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity." Proceedings of the National Academy of Sciences of the United States of America, **88**: 3720-3724.

Hamaguchi, Y., Y. Kanakura, J. Fujita, S. Takeda, T. Nakano, S. Tarui, T. Honjo and Y. Kitamura. (1987), "Interleukin 4 as an essential factor for in vitro clonal growth of murine connective tissue-type mast cells." Journal of Experimental Medicine, **165**: 268-273.

Heinzel, F. P., M. D. Sadick, B. J. Holaday, R. L. Coffman and R. M. Locksley. (1989), "Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets." J Exp Med, **169**: 59-72.

Herr, W. and J. Clarke. (1986), "The SV40 enhancer is composed of multiple functional elements that can compensate for one another." Cell, **45**: 461-470.

Ho, I. C., L. H. Yang, G. Morle and J. M. Leiden. (1989), "A T-cell-specific transcriptional enhancer element 3' of C alpha in the human T-cell receptor alpha locus." Proceedings of the National Academy of Sciences of the United States of America, **86**: 6714-6718.

Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht and R. Tjian. (1993), "Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators." Cell, **72**: 247-260.

Horikoshi, M., M. F. Carey, H. Kakidani and R. G. Roeder. (1988a), "Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions." Cell, **54**: 665-669.

Horikoshi, M., T. Hai, Y. S. Lin, M. R. Green and R. G. Roeder. (1988b), "Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex." Cell, **54**: 1033-1042.

Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka and W.E. Paul. (1982), "Identification of a T-cell derived B-cell growth factor distinct from interleukin 2." J. Exp. Med., **155**: 914-923.

Howells, G., P. Pham, D. Taylor, B. Foxwell and M. Feldmann. (1991), "Interleukin 4 induces interleukin 6 production by endothelial cells: synergy with interferon-gamma." European Journal of Immunology, **21**: 97-101.

Hu-Li, J., E. M. Shevach, J. Mizuguchi, J. Ohara, T. Mosmann and W. E. Paul. (1987), "B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting T lymphocytes." Journal of Experimental Medicine, **165**: 157-172.

Hunter, T. and M. Karin. (1992), "The regulation of transcription by phosphorylation." Cell, **70**: 375-387.

Imagawa, M., S. Osada, A. Okuda and M. Muramatsu. (1991), "Silencer binding proteins function on multiple cis-elements in the glutathione transferase P gene." Nucleic Acids Research, **19**: 5-10.

Irani, A.A., B.L. Gruber, L.D. Kaufman, M.B. Kahaleh and L.B. Schwartz. (1992), "Mast cell changes in scleroderma." Arthritis and Rhem., **35**: 933-939.

Ishizaka, T. and K. Ishizaka. (1984), "Activation of mast cells for mediator release through IgE receptors." Prog. Allergy, **34**: 188-235.

Iverson, O.H. (1989), "The cell kinetics of the inflammatory reaction. Introduction and overview." Current Topics in Pathology, **79**: 1-5.

Jackson, M. E. (1991), "Negative regulation of eukaryotic transcription. [Review]." Journal of Cell Science, **100**: 1-7

Jain, J., P. G. McCaffrey, Archer Ve Valge and A. Rao. (1992), "Nuclear factor of activated T cells contains Fos and Jun." Nature, **356**: 801-804.

Jarboe, D. L., J. S. Marshall, T. R. Randolph, A. Kukolja and T. F. Huff. (1989), "The mast cell-committed progenitor. I. Description of a cell capable of IL-3-independent proliferation and differentiation without contact with fibroblasts." Journal of Immunology, **142**: 2405-2417.

Jelinek, D.F. and P.E. Lipsky. (1988), "Inhibitory influence of IL-4 on human B cell responsiveness." J. Immunol, **141**: 164-170.

Kakkis, E., K. J. Riggs, W. Gillespie and K. Calame. (1989), "A transcriptional repressor of c-myc." Nature, **339**: 718-721.

Karim, F. D., L. D. Urness, C. S. Thummel, M. J. Klemsz, S. R. McKercher, A. Celada, Beveren C. Van, R. A. Maki, C. V. Gunther, J. A. Nye and B.J. Graves. (1990), "The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence [letter]." Genes & Development, **4**: 1451-1453.

Karray, S., A. Dautry-Varsat, M. Tsudo, H. Merle-Beral, P. Debre and P. Galanaud. (1990), "IL-4 inhibits the expression of high affinity IL-2 receptors on monoclonal human B cells." J Immunol., **145**: 1152-1158.

Kawabe, T., Y. Maeda, N. Maekawa, M. Tanaka, M. Mayumi, H. Mikawa and J. Yodoi. (1991a), "Role of PAF and cytokines in the modulation of Fc epsilon RII/CD23 expression on human eosinophils." Advances in Prostaglandin, Thromboxane, & Leukotriene Research, **21**: 989-993.

Kawabe, T., N. Maekawa, Y. Maeda, M. Hosoda and J. Yodoi. (1991b), "Induction of Fc epsilon RII/CD23 on phytohemagglutinin-activated human peripheral blood T lymphocytes. I. Enhancement by IL-2 and IL-4." Journal of Immunology, **147**: 548-553.

Keleher, C. A., C. Goutte and A. D. Johnson. (1988), "The yeast cell-type-specific repressor alpha 2 acts cooperatively with a non-cell-type-specific protein." Cell, **53**: 927-936.

Keleher, C. A., S. Passmore and A. D. Johnson. (1989), "Yeast repressor alpha 2 binds to its operator cooperatively with yeast protein Mcm1." Molecular & Cellular Biology, **9**: 5228-5230.

Kelleher, R.J., P. M. Flanagan and R. D. Kornberg. (1990), "A novel mediator between activator proteins and the RNA polymerase II transcription apparatus." Cell, **61**: 1209-1215.

Kitamura, Y., S. Go and K. Hatanaka. (1978), "Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation." Blood, **52**: 447-452.

Kitamura, Y., Y. Kanakura, J. Fujita and T. Nakano. (1987), "Differentiation and transdifferentiation of mast cells; a unique member of the hematopoietic cell family." Int J Cell Cloning, **5**: 108-121.

Kitamura, Y., H. Matsuda and K. Hatanaka. (1979), "Clonal nature of mast-cell clusters formed in W/Wv mice after bone marrow transplantation." Nature, **281**: 154-155.

Kitamura, Y., M. Yokoyama, H. Matsuda, T. Ohno and K. J. Mori. (1981), "Spleen colony-forming cell as common precursor for tissue mast cells and granulocytes." Nature, **291**: 159-160.

Klein, L. M., R. M. Lavker, W. L. Matis and G. F. Murphy. (1989), "Degranulation of human mast cells induces an endothelial antigen central to leukocyte adhesion." Proceedings of the National Academy of Sciences of the United States of America, **86**: 8972-8976.

Kopf, M., G. Le Gros, M. Bachmann, M.C. Lamers, H. Bluethmann and G. Kohler. (1993), "Disruption of the murine IL-4 gene blocks TH-2 cytokine responses." Nature, **362**: 245-248.

Kozak, M. (1988), "A profusion of controls. [Review]." Journal of Cell Biology, **107**: 1-7.

Krajewska, W. M. (1992), "Regulation of transcription in eukaryotes by DNA-binding proteins. [Review]." International Journal of Biochemistry, **24**: 1885-1898.

Kramer, H., M. Niemoller, M. Amouyal, B. Revet, Wilcken von, B. Bergmann and Hill B. Muller. (1987), "lac repressor forms loops with linear DNA carrying two suitably spaced lac operators." Embo Journal, **6**: 1481-1491.

Kuang, A. A., K. D. Novak, S. M. Kang, K. Bruhn and M. J. Lenardo. (1993), "Interaction between NF-kappa B- and serum response factor-binding elements activates an interleukin-2 receptor alpha-chain enhancer specifically in T lymphocytes." Molecular & Cellular Biology, **13**: 2536-2545.

Kurt-Jones, E., S. Hamberg, J. Ohara, W. E. Paul and A. K. Abbas. (1987), "Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness." Journal of Experimental Medicine, **166**: 1774-1787.

Lambert, P.F., B.A. Spalholz and P.M. Howley. (1987), "A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator." Cell, **50**: 69-78.

Lang, R.A. and A.W. Burgess. (1990), "Autocrine growth factors and tumourigenic transformation." Immunol. Today, **11**: 244-249.

Le-Gros, G., S. Z. Ben-Sasson, R. Seder, F. D. Finkelman and W. E. Paul. (1990), "Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells." Journal of Experimental Medicine, **172**: 921-929.

Ledermann, F., C. Heusser, C. Schlienger and G. Le Gros. (1992), "Interleukin-3-treated non-b, non-T cells switch activated B cells to IgG1/IgE synthesis." Eur. J. Immunol., **22**: 2783-2787.

Lee, H., X. Xia and Y.S. Choi. (1990), "IL-4 blocks the up-regulation of IL-2 receptors induced by IL-2 in normal human B cells." JI, **144**: 3431-3436.

Lenardo, M., J. W. Pierce and D. Baltimore. (1987), "Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility." Science, **236**: 1573-1577.

Levi-Schaffer, F., K. F. Austen, P. M. Gravallese and R. L. Stevens. (1986), "Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells [published erratum appears in Proc Natl Acad Sci U S A 1986 Oct;83(20):7805]." Proceedings of the National Academy of Sciences of the United States of America, **83**: 6485-6488.

Lewis, D. B., C. C. Yu, K. A. Forbush, J. Carpenter, T. A. Sato, A. Grossman, D. H. Liggitt and R. M. Perlmutter. (1991), "Interleukin 4 expressed in situ selectively alters thymocyte development." J Exp Med, **173**: 89-100.

Li-Weber, M., A. Eder, H. Krafft-Czepa and P.H. Krammer. (1992), "T cell specific negative regulation of transcription of the human cytokine IL-4." J. Immunol., **148**: 1913-1918.

Li-Weber, M., H. Krafft and P.H. Krammer. (1993), "A novel enhancer element in the human IL-4 promoter is suppressed by a position-independent silencer." J Immunol., **151**: 1371-1382.

Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg and M. R. Green. (1991), "Binding of general transcription factor TFIIB to an acidic activating region." Nature, **353**: 569-571.

Louie, S. W., L. M. Ramirez, A. M. Krieg, C. R. Maliszewski and G. A. Bishop. (1993), "Endogenous secretion of IL-4 maintains growth and Thy-1 expression of a transformed B cell clone." Journal of Immunology, **150**: 399-406.

Ludmila, M.C., C. Leal, D.W. Moss, R. Kuhn, W. Muller and F.Y. Liew. (1993), "Interleukin-4 transgenic mice of resistant background are susceptible to *Leishmania major* infection." Eur. J. Immunol., **23**: 566-569.

Maggi, E., P. Parronchi, R. Manetti, C. Simonelli, M. P. Piccinni, F. S. Rugiu, Carli M. De, M. Ricci and S. Romagnani. (1992), "Reciprocal regulatory effects of IFN-gamma and IL-4 on the in vitro development of human Th1 and Th2 clones." Journal of Immunology, **148**: 2142-2147.

Maire, P., J. Wuarin and U. Schibler. (1989), "The role of cis-acting promoter elements in tissue-specific albumin gene expression." Science, **244**: 343-346.

Maniatis, T., S. Goodbourn and J. A. Fischer. (1987), "Regulation of inducible and tissue-specific gene expression. [Review]." Science, **236**: 1237-1245.

Marcu, K. B., S. A. Bossone and A. J. Patel. (1992), "myc function and regulation. [Review]." Annual Review of Biochemistry, **61**: 809-860.

Masinovsky, B., D. Urdal and W.M. Gallatin. (1990), "IL-4 acts synergistically with IL-1β to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1." J Immunol., **145**: 2886-2895.

McCaffrey, P. G., C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran and A. Rao. (1993), "Isolation of the cyclosporin-sensitive T cell transcription factor NFATp." Science, **262**: 750-754.

McKnight, S. L. (1982), "Functional relationships between transcriptional control signals of the thymidine kinase gene of herpes simplex virus." Cell, **31**: 355-365.

Means, A. L. and P. J. Farnham. (1990), "Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site." Molecular and Cellular Biology, **10**: 653-661.

Meisterernst, M., M. Horikoshi and R. G. Roeder. (1990), "Recombinant yeast TFIID, a general transcription factor, mediates activation by the gene-specific factor USF in a chromatin assembly assay." Proceedings of the National Academy of Sciences of the United States of America, **87**: 9153-9157.

Mermod, N., E. A. O'Neill, T. J. Kelly and R. Tjian. (1989), "The proline-rich transcriptional activator of CTF/NF-I is distinct from the replication and DNA binding domain." Cell, **58**: 741-753.

Meyer, T. E. and J. F. Habener. (1993), "Cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. [Review]." Endocrine Reviews, **14**: 269-290.

Mican, J.M. and D.D. Metcalfe. (1990), "Arthritis and mast cell activation." J Allergy Clin. Immunol., **86**: 677-683.

Mitchell, P. J. and R. Tjian. (1989), "Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. [Review]." Science, **245**: 371-378.

Moreau, P., R. Hen, B. Wasylyk, R. Everett, M. P. Gaub and P. Chambon. (1981), "The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants." Nucleic Acids Research, **9**: 6047-6068.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin and R. L. Coffman. (1986), "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." Journal of Immunology, **136**: 2348-2357.

Mosmann, T. R. and R. L. Coffman. (1989), "TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. [Review]." Annual Review of Immunology, **7**: 145-73.

Nakabayashi, H., T. Hashimoto, M. Yasuyoshi, K. Tjong, J. Chan and T. Tamaoki. (1991), "A postition-dependent silencer plays a major role in

repressing α-fetoprotein expression in human hepatoma." Mol. Cell. Bio., **11**: 5885-5893.

Nakano, T., Y. Kanakura, T. Nakahata, H. Matsuda and Y. Kitamura. (1987), "Genetically mast cell-deficient W/W$^{\upsilon}$ mice as a tool for studies of differentiation and function of mast cells." FASEB, **46**: 1920-1923.

Nichols, M., F. Weih, W. Schmid, C. DeVack, Leutz E. Kowenz, B. Luckow, M. Boshart and G. Schutz. (1992), "Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription." EMBO J., **11**: 3337-3346.

Norman, C., M. Runswick, R. Pollock and R. Treisman. (1988), "Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element." Cell, **55**: 989-1003.

Nourbakhsh, M, K Hoffmann and H Hauser. (1993), "Interferon-β promoters contain a DNA element that acts as postition-independent silencer on the NF-κB site." EMBO J., **12**: 451-459.

Nussinov, R. (1990), "Sequence signals in eukaryotic upstream regions." Critical Reviews in Biochemistry and Molecular Biology, **25**: 185-224.

O'Shea, A., Greenfield A. and S. T. Smale. (1992), "Roles of TATA and initiator elements in determining the start site location and direction of RNA polymerase II transcription." Journal of Biological Chemistry, **267**: 1391-1402.

Ondek, B., L. Gloss and W. Herr. (1988), "The SV40 enhancer contains two distinct levels of organization." Nature, **333**: 40-45.

Orino, E., S. Sone, A. Nii and T. Ogura. (1992), "IL-4 up-regulates IL-1 receptor antagonist gene expression and its production in human blood monocytes." J Immunol, **149**: 925-931.

Pabo, C. O. and R. T. Sauer. (1992), "Transcription facotrs: Structural families and principles of DNA recognition." Annu. Rev. Biochem., **61**: 1053-1095.

Paliard, X., R. de-Waal, Malefijt, H. Yssel, D. Blanchard, I. Chretien, J. Abrams, J. de Vries and H. Spits. (1988), "Simultaneous production of IL-2, IL-4, and IFN-gamma by activated human CD4+ and CD8+ T cell clones." Journal of Immunology, **141**: 849-855.

Park, H. O. and E. A. Craig. (1989), "Positive and negative regulation of basal expression of a yeast HSP70 gene." Molecular & Cellular Biology, **9**: 2025-2033.

Paul, W.E. (1991), "Interleukin-4: A prototypic immunoregulatory lymphokine." Blood, **77**: 1859-1870.

Paul, W. E., R. A. Seder and M. Plaut. (1993), "Lymphokine and cytokine production by Fc epsilon RI+ cells. [Review]." Advances in Immunology, **53**: 1-29.

Perkins, N. D., R. M. Schmid, C. S. Duckett, K. Leung, N. R. Rice and G. J. Nabel. (1992), "Distinct combinations of NF-kappa B subunits determine the specificity of transcriptional activation." Proceedings of the National Academy of Sciences of the United States of America, **89**: 1529-1533.

Peschel, C., I. Green and W. E. Paul. (1989), "Preferential proliferation of immature B lineage cells in long-term stromal cell-dependent cultures with IL-4." Journal of Immunology, **142**: 1558-1568.

Piccinni, M. P., D. Macchia, P. Parronchi, M. G. Giudizi, D. Bani, R. Alterini, A. Grossi, M. Ricci, E. Maggi and S. Romagnani. (1991), "Human bone marrow non-B, non-T cells produce interleukin 4 in response to cross-linkage of Fc epsilon and Fc gamma receptors." Proc Natl Acad Sci U S A, **88**: 8656-8660.

Pierce, J.H., P.P. Di Fiore, S.A. Aaronson, M. Potter, J. Pumphrey, A. Scott and J.N. Ihle. (1985), "Neoplastic transformation of mast cells by Abelson-MuLV: Abrogation of IL-3 dependence by a nonautocrine mechanism." Cell, **41**: 685-693.

Pierce, J. W., A. M. Gifford and D. Baltimore. (1991), "Silencing of the expression of the immunoglobulin kappa gene in non-B cells." Molecular & Cellular Biology, **11**: 1431-1437.

Plaut, M., J. H. Pierce, C. J. Watson, Hyde J. Hanley, R. P. Nordan and W. E. Paul. (1989), "Mast cell lines produce lymphokines in response to cross-linkage of Fc epsilon RI or to calcium ionophores." Nature, **339**: 64-67.

Plum, J., M. De Smedt, G. Leclercq and B. Tison. (1990), "Inhibitory effect of murine recombinant IL-4 on thymocyte development in fetal thymus organ cultures." JI, **145**: 1066-1073.

Prendergast, GC. (1989), "DNA-binding motif." Nature, **341**: 392.

Pribnow, D. (1975a), "Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter." Proceedings of the National Academy of Sciences of the United States of America, **72**: 784-788.

Pribnow, D. (1975b), "Bacteriophage T7 early promoters: nucleotide sequences of two RNA polymerase binding sites." Journal of Molecular Biology, **99**: 419-443.

Ptashne, M. (1988), "How eukaryotic transcriptional activators work. [Review]." Nature, **335**: 683-689.

Pugh, B. F. and R. Tjian. (1990), "Mechanism of transcriptional activation by Sp1: evidence for coactivators." Cell, **61**: 1187-1197.

Pugh, B. F. and R. Tjian. (1992), "Diverse transcriptional functions of the multisubunit eukaryotic TFIID complex. [Review]." Journal of Biological Chemistry, **267**: 679-682.

Ramos, B. F., Y. Zhang, V. Angkachatchai and B. A. Jakschik. (1992), "Mast cell mediators regulate vascular permeability changes in Arthus reaction." Journal of Pharmacology & Experimental Therapeutics, **262**: 559-565.

Rao, A. (1994), "NFATp, a transcription factor required for the coordinate induction of several cytokine genes." Immunology Today, *in press*:

Renkawitz, R. (1990), "Transcriptional repression in eukaryotes. [Review]." Trends in Genetics, **6**: 192-197.

Roderiguez-Tarduchy, G., P. Malde, A. Lopez-Rivas and M. K. L. Collins. (1992), "Inhibition of apoptosis by calcium inophores in IL-3 dependent bone marrow cells is dependent upon production of IL-4." J Immunol, **148**: 1416-1422.

Romagnani, S. (1992), "Induction of T_H-1 and T_H-2 responses: a key role for the 'natural' immune response?" Immunology Today, **13**: 379-381.

Romagnani, S. (1991), "Human TH1 and TH2 subsets: doubt no more." Immunology Today, **12**: 256-257.

Ruitenberg, E. J. and A. Elgersma. (1976), "Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection." Nature, **264**: 258-260.

Sadick, M. D., N. Street, T. R. Mosmann and R. M. Locksley. (1991), "Cytokine regulation of murine leishmaniasis: interleukin 4 is not sufficient to mediate progressive disease in resistant C57BL/6 mice." Infection & Immunity, **59**: 4710-4714.

Salgame, P., J. S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R. L. Modlin and B. R. Bloom. (1991), "Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones." Science, **254**: 279-282.

Sassone, Corsi P., J. Corden, C. Kedinger and P. Chambon. (1981), "Promotion of specific in vitro transcription by excised "TATA" box sequences inserted in a foreign nucleotide environment." Nucleic Acids Research, **9**: 3941-3958.

Schaller, H., C. Gray and K. Herrmann. (1975), "Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd." Proc. Natl. Acad. Sci. USA, **72**: 737-741.

Schibler, U. and F. Sierra. (1987), "Alternative promoters in developmental gene expression. [Review]." Annual Review of Genetics, **21**: 237-257.

Schleimer, R. P., S. A. Sterbinsky, J. Kaiser, C. A. Bickel, D. A. Klunk, K. Tomioka, W. Newman, F. W. Luscinskas, M.A. Gimbrone, B. W. McIntyre and B.S. Bochner. (1992), "IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1." Journal of Immunology, **148**: 1086-1092.

Schreiber, S. L. and G. R. Crabtree. (1992), "The mechanism of action of cyclosporin A and FK506. [Review]." Immunology Today, **13**: 136-142.

Schwartz, L.B. and K.F. Austen. (1984), "Structure and function of the chemical mediators of mast cells." Prog. Allergy, **34**: 271-321.

Schwartz, L.B. (1989), "Mast cell heterogeneity with special attention to cutaneous atopic disease." Allergy, **44, suppl**: 35-40.

Seder, R.A., J. Boulay, F. Finkelman, S. Barbier, S.Z. Ben-Sasson, G. Le Gros and W.E. Paul. (1992a), "CD8+ T cells can be primed in vitro to produce IL-4." J Immunol., **148**: 1652-1656.

Seder, R. A., W. E. Paul, M. M. Davis and de St Fazekas B, Groth. (1992b), "The presence of interleukin 4 during in vitro priming determines the lymphokine-producing potential of CD4+ T cells from T cell receptor transgenic mice." Journal of Experimental Medicine, **176**: 1091-1098.

Seder, R. A., W. E. Paul, A. M. Dvorak, S. J. Sharkis, Sobotka A. Kagey, Y. Niv, F. D. Finkelman, S. A. Barbieri, S. J. Galli and M. Plaut. (1991), "Mouse splenic and bone marrow cell populations that express high-affinity Fc epsilon receptors and produce interleukin 4 are highly enriched in basophils." Proc Natl Acad Sci U S A, **88**: 2835-2839.

Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio and M. Karin. (1989), "Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes." Embo Journal, **8**: 465-473.

Sher, A., R. T. Gazzinelli, I. P. Oswald, M. Clerici, M. Kullberg, E. J. Pearce, J. A. Berzofsky, T. R. Mosmann, S. L. James and H. Morse. (1992), "Role of T-cell derived cytokines in the downregulation of immune responses in parasitic and retroviral infection. [Review]." Immunological Reviews, **127**: 183-204.

Smale, S. T. and D. Baltimore. (1989), "The "initiator" as a transcription control element." Cell, **57**: 103-113.

Smith, C. A., G. T. Williams, R. Kingston, E. J. Jenkinson and J. J. Owen. (1989), "Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures." Nature, **337**: 181-184.

Sonoda, S., T. Sonoda, T. Nakano, Y. Kanayama, Y. Kanakura, H. Asai, T. Yonezawa and Y. Kitamura. (1986), "Development of mucosal mast cells after injection of a single connective tissue-type mast cell in the stomach mucosa of genetically mast cell-deficient W/Wv mice." Journal of Immunology, **137**: 1319-1322.

Spicer, D. B. and G. E. Sonenshein. (1992), "An antisense promoter of the murine c-myc gene is localized within intron 2." Molecular & Cellular Biology, **12**: 1324-1329.

Standiford, T. J., R. M. Strieter, K. Kasahara and S. L. Kunkel. (1990), "Disparate regulation of interleukin 8 gene expression from blood monocytes, endothelial cells, and fibroblasts by interleukin 4." Biochemical & Biophysical Research Communications, **171**: 531-536.

Stenlund, A., G. L. Bream and M. R. Botchan. (1987), "A promoter with an internal regulatory domain is part of the origin of replication in BPV-1." Science, **236**: 1666-1671.

Stern, S., M. Tanaka and W. Herr. (1989), "The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16." Nature, **341**: 624-630.

Stringer, K. F., C. J. Ingles and J. Greenblatt. (1990), "Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID." Nature, **345**: 783-786.

Strobl, L. J. and D. Eick. (1992), "Hold back of RNA polymerase II at the transcription start site mediates down-regulation of c-myc in vivo." Embo Journal, **11**: 3307-3314.

Strobl, L. J., F. Kohlhuber, J. Mautner, A. Polack and D. Eick. (1993), "Absence of a paused transcription complex from the c-myc P2 promoter of the translocation chromosome in Burkitt's lymphoma cells: implication for the c-myc P1/P2 promoter shift." Oncogene, **8**: 1437-47.

Struhl, K. (1991), "Mechanisms for diversity in gene expression patterns. [Review]." Neuron, **7**: 177-1781.

Su, W., S. Jackson, R. Tjian and H. Echols. (1991), "DNA looping between sites for transcriptional activation: self-association of DNA-bound Sp1." Genes & Development, **5**: 820-826.

Swain, S. L., A. D. Weinberg, M. English and G. Huston. (1990), "IL-4 directs the development of Th2-like helper effectors." Journal of Immunology, **145**: 3796-806.

Szabo, S. J., J. S. Gold, T. L. Murphy and K.M. Murphy. (1993), "Identification of cis-acting regulatory elements controlling interleukin 4 gene expression in T cells: Roles for NF-Y and NF-AT." Mol. Cell. Biol., **13**: 4793-4805.

Tanese, N., B. F. Pugh and R. Tjian. (1991), "Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex." Genes & Development, **5**: 2212-2224.

Tara, D., D.L. Weiss and M.A. Brown. (1993), "An activation-responsive element in the murine IL-4 gene is the site of an inducible DNA-protein interaction." J Immunol., **151**: 3617-3626.

Taub, R., C. Moulding, J. Battey, W. Murphy, T. Vasicek, G. M. Lenoir and P. Leder. (1984), "Activation and somatic mutation of the translocated c-myc gene in burkitt lymphoma cells." Cell, **36**: 339-348.

Taylor, W. E. and E. T. Young. (1990), "cAMP-dependent phosphorylation and inactivation of yeast transcription factor ADR1 does not affect DNA binding." Proceedings of the National Academy of Sciences of the United States of America, **87**: 4098-4102.

Tepper, R. I., R. L. Coffman and P. Leder. (1992), "An eosinophil-dependent mechanism for the antitumor effect of interleukin-4." Science, **257**: 548-51.

Tepper, R. I., D. A. Levinson, B. Z. Stanger, Torres J. Campos, A. K. Abbas and P. Leder. (1990), "IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice." Cell, **62**: 457-467.

Tepper, R.I., P.K. Pattengale and P. Leder. (1989), "Murine interleukin-4 displays potent anti-tumor activity in vivo." Cell, **57**: 503-512.

Theoharides, T.C. (1990), "Mast cells: The immune gate to the brain." Life Sciences, **46**: 607-617.

Thornhill, M.H., U. Kyan-Aung and D.O. Haskard. (1990), "IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils." J Immunol, **144**: 3060-3065.

Todd, M. D., M. J. Grusby, J. A. Lederer, E. Lacy, A. H. Lichtman and L. H. Glimcher. (1993), "Transcription of the interleukin 4 gene is regulated by multiple promoter elements." J. Exp. Med., **177**: 1663-1674.

Toi, M., A. L. Harris and R. Bicknell. (1991), "Interleukin-4 is a potent mitogen for capillary endothelium." Biochemical & Biophysical Research Communications, **174**: 1287-1293.

Tokunaga, K., S. Hirose and Y. Suzuki. (1984), "In monkey COS cells only the TATA box and the cap site region are required for faithful and efficient initiation of the fibroin gene transcription." Nucleic Acids Research, **12**: 1543-58.

Tsai, S. Y., M. J. Tsai and B. W. O'Malley. (1989), "Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements." Cell, **57**: 443-448.

Turner, R. and R. Tjian. (1989), "Leucine repeats and an adjacent DNA binding domain mediate the formation of functional cFos-cJun heterodimers." Science, **243**: 1689-1694.

Vogel, J., M. Kress, G. Khoury and G. Jay. (1986), "A transcriptional enhancer and an interferon-responsive sequence in major histocompatibility complex class I genes." Molecular & Cellular Biology, **6**: 3550-3554.

Walsh, L. J., G. Trinchieri, H. A. Waldorf, D. Whitaker and G. F. Murphy. (1991), "Human dermal mast cells contain and release tumor necrosis factor alpha, which induces endothelial leukocyte adhesion molecule 1." Proceedings of the National Academy of Sciences of the United States of America, **88**: 4220-4224.

Wasylyk, B. (1988), "Transcription elements and factors of RNA polymerase B promoters of higher eukaryotes. [Review]." Crc Critical Reviews in Biochemistry, **23**: 77-120.

Wershil, B.K., T. Murakami and S.J. Galli. (1988), "Mast cell-dependent amplification of an immunologically nonspecific inflammatory response." J Immunol., **140**: 2356-2360.

Wershil, B. K., Z. S. Wang, J. R. Gordon and S. J. Galli. (1991), "Recruitment of neutrophils during IgE-dependent cutaneous late phase reactions in the mouse is mast cell-dependent. Partial inhibition of the reaction with antiserum against tumor necrosis factor-alpha." Journal of Clinical Investigation, **87**: 446-453.

Williams, D.E., J. Eisenman, A. Baird, C. Rauch, K.V. Ness, C.J. March, L.S. Park, U. Martin, D.Y. Mochizuki, H.S. Boswell, G.S. Burgess, D. Cosman and S.D. Lyman. (1990), "Identification of a ligand for the c-kit proto-oncogene." Cell, **63**: 167-174.

Williams, M. E., M. C. Kullberg, S. Barbieri, P. Caspar, J. A. Berzofsky, R. A. Seder and A. Sher. (1993), "Fc epsilon receptor-positive cells are a major source of antigen-induced interleukin-4 in spleens of mice infected with Schistosoma mansoni." European Journal of Immunology, **23**: 1910-1916.

Workman, J. L., I. C. Taylor and R. E. Kingston. (1991), "Activation domains of stably bound GAL4 derivatives alleviate repression of promoters by nucleosomes." Cell, **64**: 533-544.

Wright, S., L. F. Mirels, M. C. Calayag and J. M. Bishop. (1991), "Premature termination of transcription from the P1 promoter of the mouse c-myc gene." Proceedings of the National Academy of Sciences of the United States of America, **88**: 11383-11387.

Xanthoudakis, S. and T. Curran. (1992), "Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity." EMBO J., **11**: 653-665.

Xanthoudakis, S., G. Miao, F. Wang, Y. C. Pan and T. Curran. (1992), "Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme." EMBO J., **11**: 3323-3335.

Yamamoto, K. K., G. A. Gonzalez, W. Biggs and M. R. Montminy. (1988), "Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB." Nature, **334**: 494-498.

Yang-Yen, H., J. Chambard, Y. Sun, T. Smeal, T.J. Schmidt, J. Drouin and M. Karin. (1990), "Transcriptional interfernce between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction." Cell, **62**: 1205-1215.

Zhang, Y., B. F. Ramos and B. A. Jakschik. (1992), "Role of mast cells in plasma permeation due to immune injury of the skin basement membrane." Immunology, **77**: 422-427.

Collection of Papers

I. Manuscript #1

A DNASE I HYPERSENSITIVE SITE IN THE SECOND INTRON OF THE MURINE INTERLEUKIN 4 GENE DEFINES A MAST CELL SPECIFIC ENHANCER[1]

Greg Henkel[+], Deborah L. Weiss[*2], Randy McCoy[*], Thomas Deloughery[*]
David Tara[*], and Melissa A. Brown[*+3]

Departments of Medicine[*] and Microbiology/Immunology[+]
Oregon Health Sciences University, Portland, Oregon 97201

Published in Journal of Immunology, **149**: 3239-3246 (1992).

Footnotes

1. These studies were supported by grants from the Leukemia Research Foundation and National Institutes of Health grant R29 CA7992.

2. D. Weiss is supported by National Research Service award AI08353.

3. Address correspondence to: Dr. Melissa Brown, Oregon Health Sciences University, Department of Medicine- Hematology/Oncology, L586, 3181 SW Sam Jackson Park Rd., Portland, Oregon 97201.

4. Abbreviations used in this paper: PMA, phorbol myristate acetate; HS, hypersensitive site; TIS, transcription initiation site; CAT, chloramphenicol acetyl transferase

Abstract

Interleukin 4 (IL-4) is a potent immunoregulatory cytokine which exhibits extremely diverse effects on a number of target cells. Although IL-4 was originally described as a T cell-derived product, it is evident that cells of the basophil/mast cell lineage are also an important source of this cytokine. Based on their different tissue distribution, mast cell and T cell-derived IL-4 may have distinct effects on local immune responses. The physiologic production of IL-4 appears to be tightly regulated since most T and mast cells require activation to express significant levels of IL-4. In contrast, a majority of murine transformed mast cell lines constitutively express relatively high levels of IL-4. In this study, transformed mast cell lines were used as models to define *cis* acting sequences which regulate mast cell IL-4 transcription. Chloramphenicol acetyltransferase (CAT) reporter gene constructs containing 6.3 kb of 5' IL-4 flanking sequence direct relatively low CAT expression in these cells. These results indicated that additional sequences may be important in stimulating transcriptional activity of the IL-4 gene. Using DNAse I hypersensitive site analysis to define other potential IL-4 transcriptional regulatory regions, two sites were identified in the murine IL-4 gene which appear to be unique to IL-4 expressing transformed mast cells. One site defines an intronic sequence which exhibits prototypic enhancer activity in several independently derived transformed mast cell lines. This enhancer is also active in stimulated, non-transformed mast cells but not stimulated EL-4 T cells. Taken together, these data indicate that the IL-4 intronic sequence contains a mast cell specific enhancer that plays an essential role in the unregulated expression of IL-4 in transformed mast cells and may also be important in the inducible expression of IL-4 in normal mast cells.

Introduction

Interleukin 4 (IL-4) was originally defined as a T cell cytokine (1). However, it is now clear that mast cells also represent a biologically important source of IL-4 (for review see 2). Growth factor-dependent, murine mast cell lines derived from fetal liver or bone marrow express low levels of IL-4 mRNA and protein which can be upregulated by cross-linkage of the high affinity $F_c\varepsilon$ receptor ($F_c\varepsilon$ RI) or calcium ionophore stimulation (3,4). $F_c\varepsilon RI^+$, non-B, non-T cells isolated from bone marrow or spleen express IL-4 as well (5,6). These cells are presumably of mast cell/basophil lineage, produce large amounts of IL-4 when stimulated and may represent an *in vivo* equivalent of the factor-dependent mast cell lines.

The diverse activities of IL-4 suggest it plays an important role in hematopoietic cell growth and differentiation, tumor surveillance and local inflammatory responses, but little is known about the relative contribution of T cell and mast cell-derived IL-4 to these biological responses (2). The different tissue distribution of T and mast cells indicates that IL-4 production by these two types of cells may have distinct local effects. Mast cells are widely distributed in vascularized tissues in the proximity of many IL-4 target cells which contribute to inflammatory processes (7). For example, mast cells are interposed between microvascular endothelial cells in the skin, gut and lung. IL-4 secreted by mast cells may be directly responsible for several IL-4 mediated effects on endothelial cells including the induction of vascular cell adhesion molecule-1 (VCAM-1) adhesion molecules, cytokine production and cell growth (8-16). Mast cell-derived IL-4 could affect the function of cells such as monocytes, lymphocytes, eosinophils and basophils which have been recruited into an area of

localized inflammation through enhanced adhesion to the endothelium (2,13,15-17). It may also act as an autocrine factor to directly affect mast cell growth and contribute to immediate type hypersensitive responses in which mast cells are the critical effector cells (7). Fixed tissue mast cells could provide a high local concentration of IL-4 that is critical in modulating such responses. Thus, IL-4 production by tissue mast cells may play an important and distinct role in local immune/inflammatory responses compared to T cell-derived IL-4. It is important to understand the local influences which trigger IL-4 production by mast cells as well as the molecular basis of intracellular events which regulate IL-4 expression in these cells.

In contrast to normal T and mast cells which require activation to produce significant amounts of IL-4, many transformed mast cell lines express high levels of IL-4 constitutively (3). In this study these cell lines have been used as models to define *cis* acting sequences which regulate mast cell IL-4 transcription. Understanding the molecular basis of the high level of unregulated expression of IL-4 in transformed cells could also aid in delineating the critical *cis* and *trans*-acting elements which operate in the inducible transcription of the IL-4 gene in non-transformed mast cells. The results demonstrate that reporter gene constructs containing 6.3 kb of 5' IL-4 flanking sequence including the IL-4 promoter are only weakly active. However, there are unique hypersensitive sites in the IL-4 gene of transformed mast cells and one of these sites specifies the location of an intronic sequence which acts as a prototypic enhancer. In view of the low activity of the 5'IL-4/ reporter gene constructs in transformed mast cells, it is likely that this intronic sequence is an essential component of the mast cell transcription complex and plays a role in stimulating high constitutive

IL-4 expression in these cells. Interestingly, the enhancer is also active in stimulated, non-transformed mast cells but not EL-4 T cells suggesting it is mast cell specific and that T and mast cells utilize different transcriptional control elements to regulate IL-4 gene expression.

Materials and Methods

Cells. ABFTL 3 and ABFTL 2 cells were established from an IL-3 dependent mast cell line by infection with Abelson MuLV (Ab MuLV) as previously described (18). MMC34 mast cells were isolated from a murine mast cell tumor after *in vivo* infection with Ab MuLV (19). P815 is a methylcholanthrene induced transformed mast cell line (20). CFTL 12 is a non-transformed growth factor dependent (IL-3 or IL-4) mast cell line derived from fetal liver (18). EL-4 T cells (1) and WEHI 231 B cells (21) have been previously described. All cell lines were maintained in RPMI containing 10% bovine calf serum and penicillin-streptomycin. CFTL 12 cells were passaged twice weekly in the presence of 25% IL-3 containing conditioned media from WEHI 3 cells (22).

Analysis of RNA Expression. Poly A^+ mRNA was isolated from cell lines using the method described by Badely *et al* . (23). RNA samples (2 ug/lane) were electrophoresed on a 1% agarose formaldehyde gel, transferred to nitrocellulose and hybridized with an IL-4 cDNA probe which was labelled with ^{32}P using the random hexamer method (24). EL-4 cells were stimulated for 20 hours with 10 ng/ml of PMA (Sigma Chemical Company, St. Louis, MO.) and CFTL 12 cells were stimulated for 2 hours with 1 uM ionomycin (CAL Biochem, San Diego, CA.) before RNA was harvested. To verify equal amounts of RNA were loaded, the northern blot was hybridized with a murine actin cDNA probe (25).

Nuclear run-on analysis. Transcriptional activation of genes was measured by nuclear run-on analysis using the method described by

Groudine *et al.* (26). Briefly, nuclei were isolated from unstimulated CFTL 12 cells and from cells stimulated for 30 minutes and 2, 4, 6, 12 and 24 hours with 1uM ionomycin (CAL Biochem, San Diego, CA). Two x 10^7 nuclei from each time point were used as templates for *in vitro* transcription reactions performed in the presence of ^{32}P-UTP. RNA was isolated and hybridized to nitrocellulose filters containing 10 μg of linearized, immobilized and denatured murine cDNAs cloned into pGEM-3 (Promega Corp., Madison, WI) corresponding to murine IL-4 (3) and actin (25). pGEM-3 DNA was used as the negative control. These experiments were performed two times with independent preparations of nuclei.

Reporter gene constructs A murine IL-4 genomic clone, pMIL5, containing 797 bp upstream of the transcription initiation site (TIS) used in this study was the gift of Dr. Ken-ichi Arai of DNAX Research Institute (Palo Alto, CA.) and has been previously described (27). An IL-4 genomic clone containing a 10kb EcoRI fragment with approximately 6.3 kb of 5' flanking sequences was the generous gift of Drs. Eric Selsing and David Parker. To derive the -797 - +5 IL-4 reporter gene constructs which contain the IL-4 transcription initiation site (+1) but not the IL-4 ATG translation start site, an 881 bp Hind III/Rsa I fragment of pMIL5 was first cloned into the Hind III/Hinc II site of pGEM-3 (Promega Corporation). This plasmid was linearized with Eco RI to expose the 3' end of the IL-4 fragment and digested with BAL 31 exonuclease. The BAL 31 treated plasmids were then digested with Hind III and the resulting IL-4 fragments containing a 5' Hind III site and variable length 3' ends were isolated by gel electrophoresis and electroelution. The fragments were filled in with T4 DNA polymerase and subcloned into the Hinc II site of pGEM-3.

Sequencing analysis was used to identify a clone in which the ATG translation initiation site of the IL-4 gene had been removed but the (+1) site retained (-797 -+5). This IL-4 fragment was excised from pGEM-3 using Hind III and Xba I and directionally cloned into the Hind III/Xba I site of pCAT basic (-797 IL-4 pCAT) or pCAT enhancer containing an SV40 enhancer (-797 IL-4 pCAT E) (Promega Corp.). The -6300 IL-4 pCAT E construct was made by isolating a ~5.5 kb Eco RI/ Hind III fragment (located immediately 5' of -797) from a murine IL-4 genomic clone containing a 10kb Eco RI insert. The fragment was filled in with T4 DNA polymerase, Hind III linkers were added and it was cloned into the Hind III site of -797 IL-4 pCAT E. This construct contains the 5.5 kb upstream sequences in the same orientation relative to -797 sequence as the native gene.

To derive the constructs containing the intronic sequence defined by the DNAse HS studies, a 670 bp Bgl II fragment was isolated and subcloned into either the Bam HI site (distal to the SV40 promoter) or the more proximal Bgl II site of pCAT promoter (Promega Corp.) (referred to as pSV40-CAT in this manuscript)- see Figure 4. The orientation of the inserts was determined using an assymetric Pst I site located within the 670 bp fragment. Additional 3' sequences were cloned into the Bam HI site of pSV40-CAT, including an ~ 800 bp Bgl II sequence which contains exons 1 and 2, a 4kb Hind III fragment which contains exon 4 and an ~850 bp Hind III/Eco RI fragment which is located several kb downstream from exon 4 (Figure 4). The -797/670 CAT reporter gene construct was derived by subcloning the 670 Bgl II fragment into the Aat II site of a pCAT basic containing -797 to +5 of 5' murine IL-4 sequence. This sequence contains the minimal IL-4 promoter and other sequences sufficient for PMA and

ionomycin stimulated IL-4 expression in EL-4 T cells (Tara *et al.* manuscript in preparation). Positive control vectors include pCMV CAT (28) and pMoCAT (29) which contain a cytomegalovirus or Moloney MuLV promoter respectively.

Transfection and CAT assays One x 10^7 cells were transfected by electroporation with equimolar amounts (25-75 μg) of reporter gene plasmid. In experiments utilizing EL-4 T cells and CFTL 12 mast cells, which require activation to express high IL-4 levels, transfected cells were cultured overnight; EL-4 cells were then stimulated with 10 ng/ml of PMA and cell extracts were prepared 24 and 48 hours later as described (30). CFTL 12 cells were stimulated with 1 μM ionomycin and cell extracts prepared after 6-8 and 24 hours. ABFTL 3, ABFTL 2, MMC34, P815 and WEHI 231 cell extracts were harvested 24 and 48 hours after electroporation. CAT assays were performed using equal amounts of extract in a scintillation diffusion assay based on the method described by Neumann *et al.* (31). Briefly, extracts were heated at 65°C for 10 minutes to inactivate cellular deacetylases. Fifty μl of extract was mixed with 200 ul of 1.25 mM chloramphenicol and 0.1 μCi 3H-acetyl CoA (NEN, Boston, MA.) (final acetyl CoA concentration in the reaction is 0.02 mM). This reaction was overlayed with 2 ml of Econofluor 2 scintillation fluid (NEN) and the diffusion of the acetylated chloramphenicol at 6 hours was measured by scintillation counting.

To control for differences in transfection efficiency within experiments, all cells except EL-4 were co-transfected with a b-galactosidase expression vector (32) and CAT activity was normalized to b-gal activity using an assay previously described (33). In experiments with

EL-4 cells, b-gal activity was not detectable. Therefore, protein determinations in cell extracts were performed using a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA.) based on the method of Bradford (34) and CAT activity was normalized to protein levels. CAT activities reported for ABFTL 3, EL-4 and CFTL 12 cells are representative of at least six independent transfection experiments. Experiments using MMC34, P815, WEHI 231 or ABFTL 2 cells were performed at least three times.

DNase I hypersensitive site analysis. The isolation of nuclei, digestion with DNase I and purification of DNA were performed based on the method described by Siebenlist *et al.* (35). Briefly, nuclei were isolated from cells after detergent lysis with Nonidet P-40, suspended at a concentration of 2 x 10^7/ml and digested with DNase I (Bethesda Research Laboratories, Rockville, MD.) at concentrations ranging from 0.5 - 120U/ml for 5 minutes at room temperature. DNA was isolated, subject to restriction endonuclease digestion and Southern blot analysis was performed using standard techniques (36). To induce IL-4 expression in DNAse I experiments, EL-4 cells were stimulated with 10 ng/ml of PMA for 6, 12, 18 and 24 hours and CFTL 12 mast cells were stimulated with 1 uM ionomycin for 30 minutes, 2 hours and 6 hours and 12 hours. Analyses with ABFTL 3, P815 and WEHI 231 cells were performed with unstimulated cells.

Hybridization probes. A Hind III/Rsa I fragment containing 797 bp upstream of the transcription initiation site and a portion of exon I of murine IL-4 gene was used in most DNAse I experiments. In some experiments, a 373 bp Rsa I fragment from an IL-4 cDNA clone was used (3).

Results

Expression of IL-4 mRNA in Normal and Transformed Mast Cells. Although most T and mast cell lines require activation to express significant levels of IL-4, it was previously demonstrated that many transformed mast cell lines constitutively express high steady state IL-4 mRNA levels (3, 4). To confirm the IL-4 expression phenotype of the cell lines used in these experiments, steady state IL-4 mRNA content was analyzed (Figure 1A). High basal levels of IL-4 mRNA are detected in the transformed mast cell lines P815, MMC34 and ABFTL 3 (Figure 1A, lanes 3,5 and 7). This expression is comparable to levels observed in stimulated EL-4 T cells (lane 2) and stimulated CFTL 12 mast cells (lane 9). ABFTL 2 (lane 4), a transformed mast cell line, WEHI 231 (lane 6), a B cell line, and EL-4 cells (lane 1), do not express detectable basal levels of IL-4 mRNA. Unstimulated CFTL 12 mast cells express low levels of IL-4 message (lane 8).

An increase in steady state mRNA levels in activated cells could reflect both transcriptional and post-transcriptional mechanisms. To determine how increased transcription contributes to high steady state IL-4 levels in stimulated CFTL 12 mast cells, nuclear run-on analysis was performed. Ionomycin induces a significant increase in the transcriptional activity of the IL-4 gene which is maximal at 30 minutes post-stimulation and decreases to basal levels by 6 hours (Figure 1B). These data demonstrate that the increase in IL-4 mRNA in stimulated CFTL 12 mast cells is regulated, at least in part, by an increase in the transcriptional activity of the IL-4 gene.

Activity of 5' IL-4 CAT reporter gene constructs in transformed mast cells To identify sequences that control IL-4 transcription, 5' flanking regions were tested in ABFTL 3 transformed mast cells in transient transfection assays using a variety of CAT reporter gene constructs. A representative experiment is shown in Table I. Only low levels of CAT activity could be detected in extracts from cells transfected with constructs containing up to 6.3 kb of 5' IL-4 sequence. (The presence of an SV40 enhancer {pCAT E constructs} increased the level of CAT activity detected but it still was not commensurate with the high levels of IL-4 mRNA observed in these cells). The -6300 - +5 IL-4 constructs were highly active in stimulated EL-4 T cells and CFTL 12 and 15 mast cells (Tara *et al.*, and Weiss *et al.* manuscripts in preparation). The low levels of CAT activity in ABFTL 3 cells cannot be attributed to low transfection efficiency or inability to express CAT. The pMo CAT control vector directed high levels of CAT expression (Table 1) and significant activity from the co-transfected b-gal expression vector was detected in all cell extracts (data not shown).

Chromatin structure of IL-4 gene in IL-4 expressing T and mast cells. The inability to detect high CAT expression suggested that regions outside the 6.3 kb upstream region may contribute to the high basal level of IL-4 mRNA expressed in transformed mast cells. To identify these potential regulatory regions, DNAse I hypersensitive site analysis was performed. This assay reveals sites in the chromatin of transcriptionally active or potentially active genes based on their unusual susceptibility to limiting concentrations of DNAse I (37). Such sites have been shown to define important transcriptional control regions in genes such as IL-2, γIFN,

globin and CD2 (38-41). DNAse I HS site analysis of Hind III digested DNA isolated from several cell lines is shown in Figure 2A. A 5.5 kb band appears in all lanes and corresponds to the IL-4 Hind III restriction fragment as diagrammed in Figure 2B. In DNAse I treated nuclei from ABFTL 3 and P815 transformed mast cells, a sub-band of approximately 2.2 kb is present. This sub-band represents a DNAse I hypersensitive site which maps to a region in the second intron of the IL-4 gene ~1.4 kb downstream from the transcription initiation site (Figure 2B). This site was not detected in nuclei from EL-4 cells or CFTL 12 cells assayed at various times post stimulation or in nuclei from unstimulated EL-4, CFTL 12 or WEHI 231 cells (data not shown).

DNAse I HS analysis of Eco RI digested DNA from all cells revealed a 10 kb band corresponding to the expected IL-4 genomic Eco RI fragment (Figures 3A and 3B). ABFTL 3 and P815 cells revealed a DNAse I specific sub-band of approximately 3.1 kb (Figure 3A). The 3.1 kb sub-band cannot be the result of a DNAse I site at 3.1 kb from either Eco R1 site because the probe does not cover that region. Thus, we conclude that this sub-band is the result of two distinct DNAse I sites: the site in the second intron defined by the Hind III analysis and a second site approximately 1.6 kb upstream of the transcription initiation site (Figure 3B). The 3.1 kb sub-band was not seen in any of the other cells tested since its detection is dependent on the presence of the intronic site that was only found in the transformed mast cells. However, using an IL-4 cDNA as a hybridization probe with DNA from EL-4 cells or CFTL 12 cells, no evidence of 5' sites was found. Other gene fragments derived from regions further 5' or 3' of the IL-4 gene coding regions were not useful as hybridization probes since they resulted in non-specific hybridization of the

Southern blots, indicating they probably contained significant repetitive sequences.

A weaker 8.2 kb sub-band that was present only in DNAse I treated samples of ABFTL 3 cells was also observed. This band must correspond to a site extremely 5' of the IL-4 gene at approximately -4.5 kb since a band indicating an extreme 3' site was not detected in Hind III digests. Other sub-bands were also detected, particularly in P815 cells but these were also found in samples that were not treated with DNAse I suggesting that they resulted from endogenous endonuclease activity.

The intronic DNAse I HS site defines a transcriptional enhancer. Since DNase I HS sites have been shown to correlate with important regulatory regions in a number of genes (37-42), it was possible that the transformed mast cell HS sites define important IL-4 control regions. The -6300 - +5 IL-4 reporter gene constructs contain the sequences comprising the 5' DNAse I HS sites but were not active. If these HS sites define important IL-4 regulatory regions, other interacting sequences outside the -6.3 kb region are required for activity. Therefore, we focused on analyzing the region defined by the intronic DNAse HS site. Four discrete 3' regions of the murine IL-4 gene were subcloned into pSV40-CAT which contains an SV40 promoter (Figure 4). The ability of these sequences to enhance SV40 promoter driven CAT expression was then measured. A 670 bp Bgl II sequence located in the second intron significantly enhances SV40 driven CAT expression in three independently derived IL-4 expressing transformed mast cell lines: ABFTL 3, MMC34 and P815 (Figure 5). This fragment is comprised of sequences very close to the estimated position of the intronic DNAse HS site. The 800 bp, 850 bp and 4 kb

fragments (see Figure 4) were not active in several independent transfection experiments (data not shown). It has been difficult to subclone and therefore test the sequences immediately 3' of the 670 bp Bgl II fragment. Since this region contains sequences which may comprise or at least flank the HS site, its potential contribution to IL-4 gene regulation cannot be ruled out. However, these data indicate that the intronic DNAse I HS site specifies the location of an IL-4 regulatory element. The high activity of the 670 bp Bgl II sequence in unstimulated transformed mast cells also suggests it plays a role in constitutive IL-4 production.

The enhancer activity of the intronic sequence is position and orientation independent. Prototypic enhancers are defined by their ability to act in an orientation and position independent manner (43). To determine whether the 670 bp Bgl II intronic sequence demonstrates these features, the activity of pSV40-CAT/670 constructs in which the position and orientation of the enhancer were varied were compared to pSV40-CAT. Although there are modest but consistent position-dependent effects, the Bgl II intronic sequence significantly enhances CAT activity in transformed mast cells regardless of its orientation or position (Figure 6).

The intronic sequence enhances IL-4 promoter driven CAT expression in transformed mast cells. To evaluate the ability of the 670 bp Bgl II sequence to enhance IL-4 promoter driven CAT expression, a construct containing the 670 bp sequence cloned immediately upstream of -797 to +5 IL-4 5' flanking sequences (670/-797 IL-4 promoter) was tested. This construct reproducibly directed increased CAT expression (8-10 fold) relative to -797 IL-4 pCAT in ABFTL 3 cells (Figure 7). These results

indicate that the intronic sequence can act on the IL-4 promoter as well as the heterologous SV40 promoter to enhance CAT transcription and strengthens our conclusion that this sequence has the features of a prototypic enhancer element.

The intronic sequence contains a mast cell specific enhancer The enhancer activity of the 670 bp Bgl II sequence was next evaluated in cell lines which produce high IL-4 levels only when activated. The ability of the enhancer to act with the SV40 and IL-4 promoter in CFTL 12 mast cells and EL-4 T cells was compared (Figures 8A and 8B). The sequence moderately but reproducibly enhanced expression of pSV40 CAT (1.5-2.5 fold) in ionomycin-stimulated CFTL 12 mast cells although CAT levels were very low (Figure 8A). Little detectable enhancer activity was detected in unstimulated cells. CAT expression from 670/-797 IL-4 pCAT was significantly higher (5-8 fold) relative to -797 IL-4 pCAT (Figure 8A) in ionomycin-stimulated cells. In addition, the enhancer was slightly active in unstimulated cells as well. The activity in unstimulated cells is consistent with the fact that low levels of basal IL-4 expression are observed.

The 670 bp fragment was unable to enhance CAT expression above the level of pSV40-CAT and -797 IL-4 pCAT in unstimulated and PMA-stimulated EL-4 cells (Figure 8B). The enhancer was also inactive in cell lines which do not express IL-4 including WEHI 231 B cells and ABFTL 2 mast cells (data not shown). These data suggest that the enhancer is mast cell specific and may play a role in stimulating IL-4 transcription in both normal and transformed mast cells.

Discussion

IL-4 is a pleiotropic cytokine whose physiologic expression is tightly regulated (2). In most T cells and mast cells examined, high levels of IL-4 mRNA and protein are produced only after activation. However, a group of transformed murine mast cells have been identified which exhibit high constitutive expression of IL-4 (3). ABFTL 3, MMC34 and P815 are representative of this unique class of IL-4 producing cells. ABFTL 3 was derived from IL-3 dependent mast cell lines by *in vitro* infection with Abelson MuLV (18). MMC34 cells resulted from an *in vivo* Ab MuLV infection (19) and P815 is a methylcholanthrene-induced tumor (20). In contrast to non-transformed mast cell lines, these cell lines do not require exogenous IL-3 for growth and do not express IL-3. However, they do express high levels of IL-4 mRNA and IL-4 protein constitutively (3). There is no evidence of IL-4 gene rearrangements which could cause dysregulation of IL-4 expression (3). The role of IL-4 as a mast cell growth factor, its high constitutive production and the lack of IL-3 production by these cells suggested that IL-4 may be acting as an autocrine growth factor (3,5). Experiments using anti-IL-4 antibody to block the *in vitro* growth of these cells were unsuccessful (3). However, antisense oligonucleotides directed to the translation start site of the IL-4 mRNA block ^{3}H-thymidine uptake in ABFTL 3 cells (M. Brown, unpublished results). Such results are consistent with an autocrine role for IL-4, perhaps via an internal receptor as was described in the autocrine growth of IL-3 producing hematopoietic cell lines (44).

In this study, the molecular basis of high basal IL-4 expression in transformed mast cells was examined. Since most gene regulatory

sequences are located upstream of of the transcription initiation site, it was suprising that 6.3 kb of 5' IL-4 sequence is able to direct only low levels of CAT reporter gene transcription in transformed mast cells. Therefore, DNase I HS analysis was performed to identify additional regulatory elements which may be required for high constitutive expression. At least two HS sites in the IL-4 gene were defined which are unique to IL-4 expressing transformed mast cells. One site, located in the second intron, specifies the location of a sequence contained in a 670 bp Bgl II fragment which acts as a prototypic enhancer: it is orientation and position independent and acts in concert with a heterologous SV40 promoter as well as the IL-4 promoter in unstimulated transformed mast cells. In view of the low activity of the 5' IL-4 reporter gene constructs, it is likely that the intronic sequence described in this report is a critical component of the IL-4 transcription complex in these cells and contributes to high basal IL-4 expression.

The enhancer sequence was consistently more active with the SV40 promoter (enhanced 9-31 fold over SV40 promoter alone) than with -797 - +5 of 5' IL-4 sequence (5-9 fold enhancement) in transformed mast cells. One interpretation of this observation is that other *cis*-acting IL-4 sequences may be required to fully reconstitute the transcriptional activity *in vitro*. Sequences in the vicinity of the 5' HS site (~1.6 kb and ~4.5 kb upstream of the TIS) as well as those immediately 3' of the intronic DNAse site which have not yet been assayed are potential candidates for such a collaborative role. We are currently examining other regions of the IL-4 gene to identify the sequences and associated transcription factors that may interact with the intronic enhancer including a -6300 - +5/670 construct which contains the 5' HS sites. The contribution of post-transcriptional

regulatory mechanisms to high steady state levels of IL-4 mRNA have not yet been examined and may be important as well.

Coincident with its ability to transform myeloid lineage cells, Ab MuLV infection is associated with the activation of multiple growth factor genes including IL-3, GM-CSF and IL-6 (45). Since a majority of the IL-4 expressing transformed mast cells are derived by Abelson MuLV infection of growth factor-dependent cells, it is possible that viral gene products such as the *v-abl* oncogene are involved in stimulating constitutive IL-4 gene expression in these cells. Our results cannot formally eliminate this possibility. However, P815 is a methylcholanthrene-induced mastocytoma which was not overtly infected with MuLV and yet appears to be regulated similarly to the Ab MuLV transformed lines. In addition, *v-abl* expression is not associated with IL-4 production in several mast cell lines of similar derivation to ABFTL 3 including the ABFTL 2 cell line reported in this study. Thus, infection by Ab MuLV and expression of viral gene products in these cells is not sufficient to activate IL-4 gene expression.

In addition to its likely role in stimulating high constitutive IL-4 expression in transformed mast cells, the intronic enhancer appears to play a role in the more physiologic, inducible expression of IL-4 demonstrated by the normal CFTL 12 mast cell line. The intronic sequence, in concert with the SV40 promoter, enhanced CAT expression by 1.5-2.5 fold relative to pSV40 CAT in ionomycin-stimulated CFTL 12 cells. Significantly, the intronic sequence enhances IL-4 promoter driven CAT expression by 6-8 fold in stimulated CFTL 12 mast cells and thus collaborates better with the IL-4 promoter than with the SV40 promoter. In fact, as in the transformed mast cells, the -797 - +5 IL-4 constructs are essentially

negative without the intronic sequence. These results strongly imply that the intronic enhancer is essential in regulating the inducible transcription of IL-4 in non-transformed mast cells as well. The DNase I HS site which originally defined this enhancer in transformed mast cells is not detected in CFTL 12 cells. This may be due to the fact that the altered chromatin configuration detected by HS site analysis occurs in activated CFTL 12 cells at a time post-stimulation which was not assayed. Alternatively, the lack of a HS site could reflect a less dominant role for this sequence in IL-4 transcription in normal mast cells compared to transformed cells.

The intronic sequence is not active with either the SV40 or IL-4 promoter in unstimulated or stimulated EL-4 T cells. It is also inactive in cells which do not produce IL-4 such as WEHI 231 B and ABFTL 2 mast cell lines. Thus, the activity of the intronic enhancer appears to be limited to IL-4 producing cells of mast cell lineage. These results suggest that the enhancer is mast cell specific although additional T and mast cell lines need be analysed to confirm this supposition.

It will be important to determine the way in which the intronic enhancer sequence contributes to high basal IL-4 expression in transformed mast cells and whether it acts differently in regulating inducible mast cell expression. High basal expression in transformed cells may be influenced in several ways by the intronic regulatory elements defined here. Transformed mast cells may express unique DNA binding proteins that are not found in non-transformed cell lines. Alternatively, there may be a quantitative difference in the amount of specific transcription factors that act on this sequence or a transformed cell specific modification of a common DNA binding protein which results in constituive activity. Studies are underway to further characterize this sequence and determine the

nature of its interaction with associated DNA binding proteins in both growth factor-dependent and transformed mast cells.

Acknowledgements

We thank Drs. Jim Miller, Linda Shapiro and Grover Bagby for their helpful discussions of this work and gratefully acknowledge the excellent technical assistance of Ms. Karen Wisegarver.

References

1. **Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W.E. Paul.** 1982. Identification of a T-cell derived B-cell growth factor distinct from interleukin 2. *J. Exp. Med.* 155:914.

2. **Paul, W.E.** 1991. Interleukin-4: A prototypic immunoregulatory lymphokine. *Blood.* 77:1859.

3. **Brown, M.A.,J.H. Pierce, C.J. Watson, J. Falco, J.N. Ihle, and W.E. Paul.** 1987. B cell stimulatory factor-1/Interleukin mRNA is expressed by normal and transformed mast cells. *Cell* 50:809.

4. **Plaut, M., J. H. Pierce, C.J. Watson, J. Hanley-Hyde, R.P. Nordon, and W.E. Paul.** 1989. Mast cell lines produce lymphokines in response to cross-linkage of $F_C\varepsilon RI$ or calcium ionophores. *Nature* 339:64.

5. **Ben-Sasson, S.Z., G. LeGros, D.H. Conrad, and R.D. Finkelman.** 1990. Cross-linking Fc receptors stimulate splenic non-B, non-T cells to secrete interleukin 4 and other lymphokines. *Proc. Natl. Acad. Sci.* 87:1421-1425.

6. **Piccinni, M. P., D. Macchia, P. Parronchi, M.G. Giudizi, D. Bani, R. Alterini, A. Grossi, M. Ricci, E. Maggi and S. Romagnani.** 1991. Human bone marrow non-B, non-T cells produce IL-4 in response to cross-linkage of $F_C\varepsilon$ and $F_C\gamma$ receptors. *Proc. Natl. Acad. Sci.* 88:8656.

7. **Czarnetzki, B.M.** 1987. Mechanisms and Mediators in Urticaria. *Sem. Dermatol.* 6:272.

8. **Issekutz, T.B.** 1990. Effects of six different cytokines on lymphocyte adherence to microvascular endothelium and in vivo lymphocyte migration in the rat. *J. Immunol.* 144: 2140.

9. **Masinovsky, B., D. Urdal and W. M. Gallitin.** 1990. IL-4 acts synergistically with IL-1b to promote lymphocyte adhesion to microvascular endothelium by induction of vascular cell adhesion molecule-1. *J. Immunol.* 145: 2886.

10. **Schleimer, R.P., S. A. Sterbinsky, J. Kaiser, C.A. Bickel, D.A. Klunk, K. Tomioka, W. Newman, F.W. Luscinskas, M.A. Gimbrone, Jr., B.W. McIntyre and B.S. Bochner.**1992. IL4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium: Association with VCAM-1. *J. Immunol.* 148:1086.

11. **Howells, G., P. Pham, D. Taylor, B. Foxwell and M. Feldmann.** 1991. Interleukin 4 induces interleukin 6 production by endothelial cells: Synergy with interferon gamma. *Eur. J. Immunol.* 21: 97-101.

12. **Thornhill, M. H. and D.O. Haskard.** 1990. IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor or IFN-gamma. *J. Immunol.* 145:865-872.

13. **Standiford, T.J., R. M. Strieter, K. Kasahara and S. L. Kunkel.** 1990. Disparate regulation of interleukin 8 gene expression from blood monocytes, endothelial cells, and fibroblasts by interleukin 4. *Biochem. Biophys. Res. Commun.* 171: 531-536.

14. **Toi, M., A.L. Harris and R. Bicknell.** 1991. Interleukin-4 is a potent mitogen for capillary endothelium. *Biochem. Biophys. Res. Commun.* 174: 1287-1293.

15. **Crawford, R.M., D.S. Finbloom, J. Ohara, W.E. Paul, and M.S. Meltzer.** 1987. B cell stimulatory factor-1 (interleukin 4) activates macrophages for increased tumoricidal activity and expression of Ia antigens. *J. Immunol.* 139:135-141.

16. **Ralph, P; I. Nakoinz, and D.Rennick.** 1988. Role of interleukin-2, interleukin-4, and alpha, beta, and gamma interferon in stimulating macrophage antibody-dependent tumoricidal activity. *J. Exp. Med.* 167:712-717.

17. **Littman, B.H., F. Dastvan, F.Frank, P.L. Carlson, and K.M. Sanders.** 1989. Regulation of monocyte/macrophage C2 production and HLA-DR expression by IL-4 (BSF-1) and IFNγ. *J. Immunol.* 142: 520-525.

18. **Pierce, J. H., PP. DiFiore, S.A. Aaronson, M. Potter, J. Pumphrey, A. Scott, and J. N. Ihle.** 1985 Neoplastic transformation

of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. *Cell* 41:685.

19. **Barsumian, E.L., A. McGivney, L. Basciano, and R. Siraganian.** 1985. Establishment of four mouse mastocytoma cell lines. *Cell. Immunol.* 90:131.

20. **Dunn, T.B. and M. Potter.** 1957. A transplantable mast cell neoplasm in the mouse. *J. Natl. Cancer Inst.* 18:587.

21. **Gutman, G.A., N.L. Warner, and L.L. Lanier.**1981. Immunoglobulin production by murine B-lymphoma cells. *Clin. Immunol. and Immunopath.* 18:230.

22. **Ralph, P. M.A.S. Moore, and K. Nilsson.** 1976. Lysozyme synthesis by established human and murine histiocytic lymphoma lines. *J. Exp. Med.* 143: 1528.

23. **Badely, J.E., G.A. Bishop, T. St. John, and J.A. Frelinger.** 1988. A simple, rapid method for the purification of Poly A^+ RNA. *Biotechniques* 6:114.

24. **Feinberg, A. P. and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.

25. **Alonso, S., A. Minty, Y. Bourlet, and M. Buckingham.** 1986. Comparison of Three Actin-Coding Sequences in the Mouse; Evolutionary Relationships between the actin genes of warm-blooded vertebrates. *J. Mol. Evol.* 23:11.

26. **Groudine, M., M. Peretz, and H. Weintraub**. 1981. Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol. Cell Biol.* 1:281.

27. **Otsuka, T., Villaret, D. T. Yokota, Takebe, Y. F. Lee, N. Arai, and K. Arai.** 1987. Structural analysis of the mouse chromosomal gene encoding interleukin 4 which expresses B cell, T cell and mast cell stimulating activities. *Nuc. Acids Res.* 15:333.

28. **Foecking, M.K. and H.Hofstetter**. 1986. Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene* 45:101.

29. **Linney, E., B. Davis, J. Overhauser, E. Chao, and H. Fan**. 1984. Non-function of a Moloney murine leukaemia virus regulatory sequence in F9 embryonal carcinoma cells. *Nature* 308:470.

30. **Gorman, C.M., L.F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044.

31. **Neumann, J.R., C.A. Morency, and K.O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. *Biotechniques* 5:444.

32. **Price, J., D. Turner, and C. Cepko.** 1987. Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA* 84:156.

33. **Norton, P.A. and J.M. Coffin.** 1985. Bacterial b-galactosidase as a marker of Rous Sarcoma Virus Gene Expression and Replication. *Mol. and Cell. Biol.* 5:281.

34. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72:234.

35. **Siebenlist, U., L. Hennighausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt's lymphoma. *Cell* 37:381.

36. **Southern, E.M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.

37. **Gross, D.S. and W. T. Garrard.** 1988. Nuclease hypersensitive sites in chromatin. *Ann. Rev. Biochem.* 57:159.

38. **Siebenlist, U., D.B. Durand Durand, P. Bressler, N.J. Holbrook, C.A. Norris, M. Kamoun, J.A. Kant, and G.R. Crabtree.** 1986. Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol

acetyltransferase gene during activation of T cells. *Mol. and Cell. Biol.* 6:3042.

39. **Hardy, K.J., B. Manger, M. Newton, and J. Stobo.** 1987. Molecular events involved in regulating human interferon-γ gene expression during T cell activation. *J. Immunol.* 138:2353.

40. **Ciccarone, V.C., J. Chrivia, K.J. Hardy, and H.A. Young.** 1990. Identification of enhancer-like elements in human IFN -γ genomic DNA. *J. Immunol.* 144:725.

41. **Lowrey, C.H., D.M. Bodine, and A.W. Nienhuis.** 1992. Mechanism of DNase I hypersensitive site formation within the human globin locus control region. *Proc. Natl. Acad. Sci. U.S.A.* 89:1143.

42. **Lake, R.A., D. Wotton, and M.J. Owen.** 1990. A 3' transcriptional enhancer regulates tissue specific expression of the human CD2 gene. *EMBO J.* 9:3129.

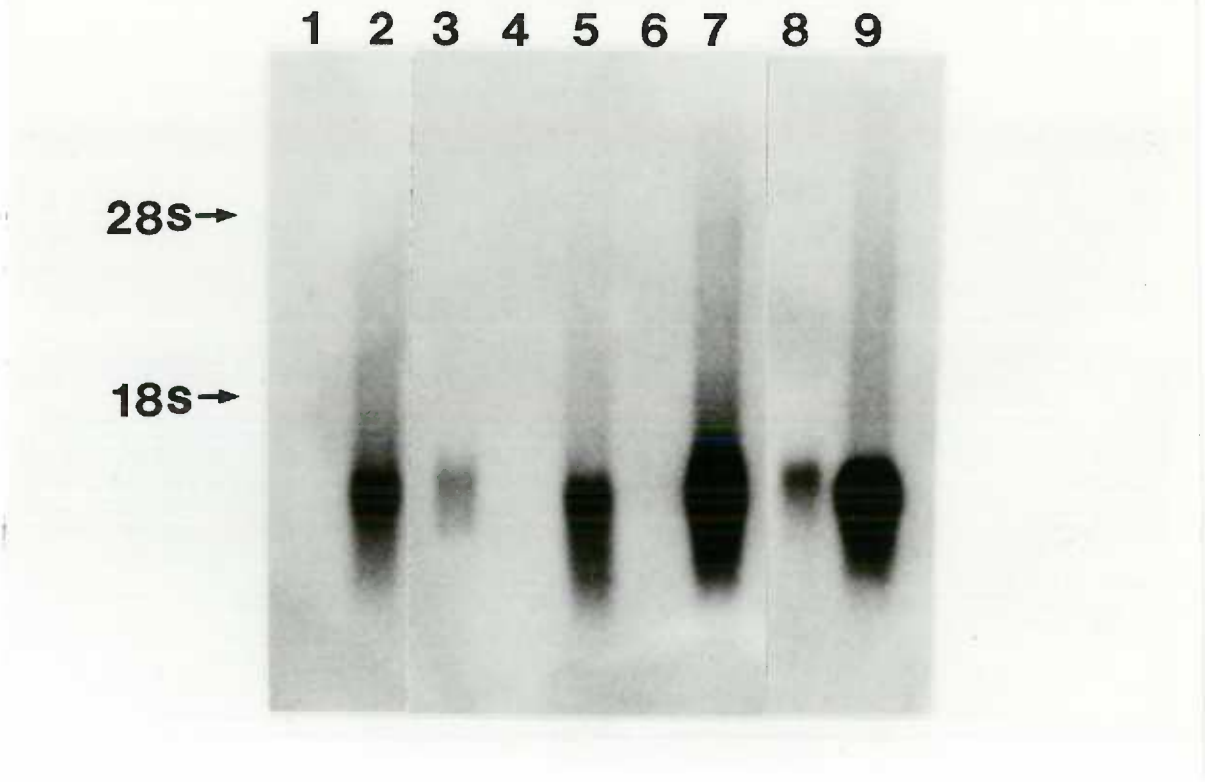
43. **Muller, M.M., T. Gerster, and W. Schaffner.** 1988. Enhancer sequences and the regulation of gene transcription. *Eur. J. Biochem.* 176:485.

44. **Browder, T.M., J.S. Abrams, P.M.C. Wong, and A.W. Nienhuis.** 1989. Mechanism of autocrine stimulation in hematopoietic cells producing interleukin-3 after retrovirus-mediated gene transfer. *Mol and Cell. Biol.* 9:204.

45. **Humphries, R.K., S. Abraham, G. Krystal, P. Landsdorp, F. Lemoine, and C.J. Eaves.** 1988. Activation of multiple hemopoietic growth factor genes in Abelson virus-transformed myeloid cells. *Exp. Hematol.* 16:774.

Figure 1A: Analysis of steady state IL-4 mRNA expression. 2 μg of Poly $(A)^+$ RNA was analyzed on northern blots which were hybridized with a ^{32}P labelled IL-4 cDNA probe. Lane: 1) unstimulated EL-4 2) PMA stimulated (10 ng/ml for 20 hours) EL-4 3) P815 4) ABFTL 2 5) MMC34 6) WEHI 231 7) ABFTL 3 8) unstimulated CFTL 12 9) ionomycin stimulated (1 μM for 2 hours) CFTL 12

Figure 1B: Transcriptional activation of IL-4 gene expression in CFTL 12 mast cells. Nuclei were isolated from unstimulated CFTL 12 mast cells (0) and cells stimulated with 1 μM ionomycin for various times (indicated in hours). *In vitro* transcription reactions were performed in the presence of ^{32}P-UTP and hybridized to filters containing equal amounts of pGEM-3, a murine IL-4 cDNA or murine β-actin cDNA.


1 2 3 4 5 6 7 8 9
28s
18s

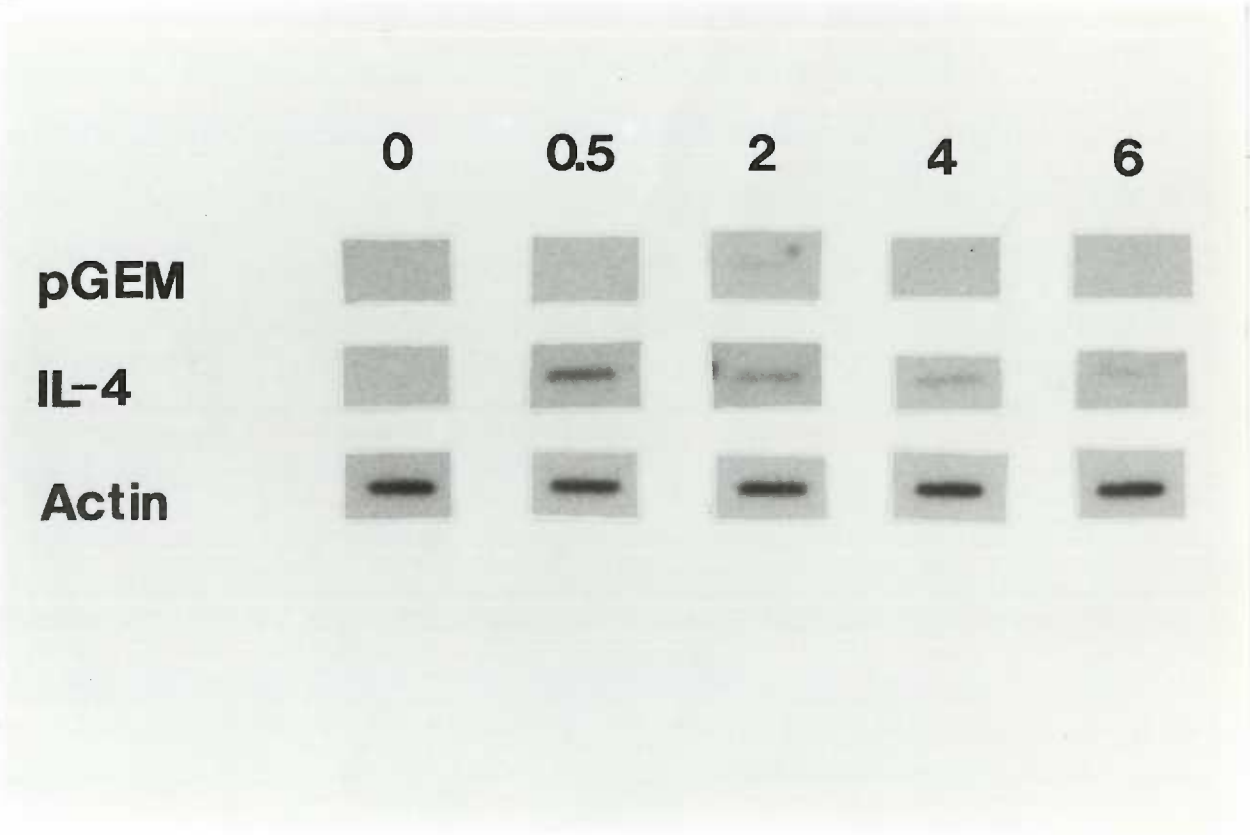

0 0.5 2 4 6
pGEM
IL-4
Actin

TABLE I

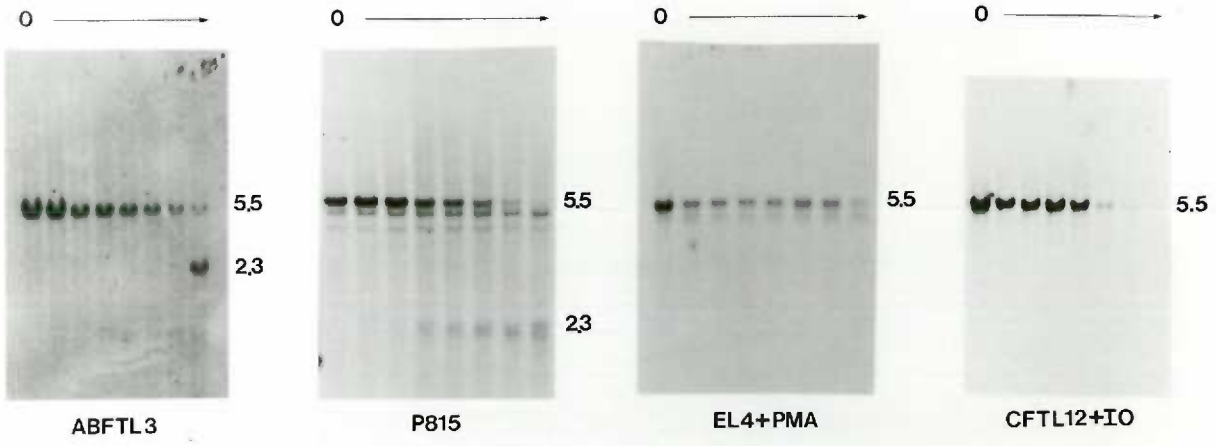
IL-4 5' FLANKING SEQUENCE REPORTER GENE CONSTRUCTS ARE ONLY WEAKLY ACTIVE IN TRANSFORMED MAST CELLS WHICH EXPRESS HIGH BASAL LEVELS OF IL-4

	ABFTL 3
Plasmid	cpm
Control	1132
pMo CAT	81867
pCAT promoter	5636
-797 IL-4 pCAT	**1860**
-797 IL-4 pCAT E	**8622**
-6300 IL-4 pCAT E	**6004**

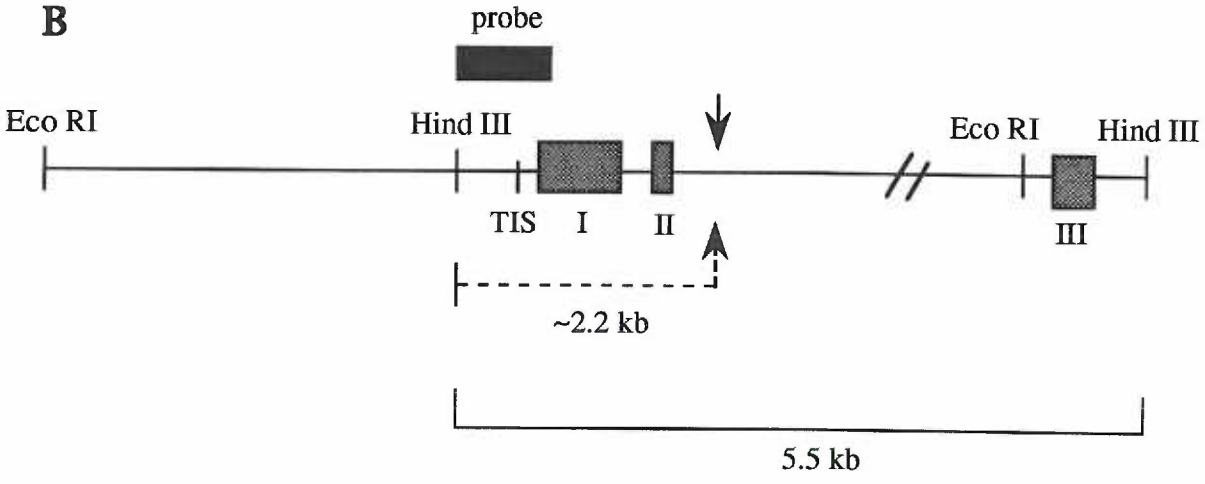
Representative experiment testing the activity of 5' IL-4 CAT reporter gene constructs in ABFTL 3 transformed mast cells. Cells were transfected by electroporation with equimolar amounts of the indicated reporter gene constructs. CAT activity was assayed 48 hours after transfection. In all experiments, cells were co-transfected with a β-galactosidase expression vector and CAT cpms were normalized to the level of β-gal activity. pCAT enhancer contains no promoter and serves as the negative control.

Figure 2: DNAse I hypersensitive site analysis of Hind III digested chromosomal DNA. A) Southern blot analyses of DNAse I treated DNA from ABFTL 3 and P815 transformed mast cells and stimulated EL-4 T cells and CFTL 12 mast cells. Nuclei were isolated from cells and DNAse I treatment performed as described (increasing DNAse concentrations indicated by arrows above each blot). Analyses shown with ABFTL 3 cells and P815 cells were performed on nuclei from unstimulated cells. EL-4 cells were stimulated for 20 hours with 10 ng/ml of PMA and CFTL 12 cells were stimulated for 30 minutes with 1μM ionomycin (IO). The sizes of resulting genomic fragments in kb are indicated to the right. B) Localization of the HS sites on the murine IL-4 chromosomal map based on Hind III digestion of DNAse I treated chromosomal DNA. A Hind III/Rsa I fragment containing IL-4 5' flanking sequences and part of exon I was used as the hybridization probe in these experiments.

A



B

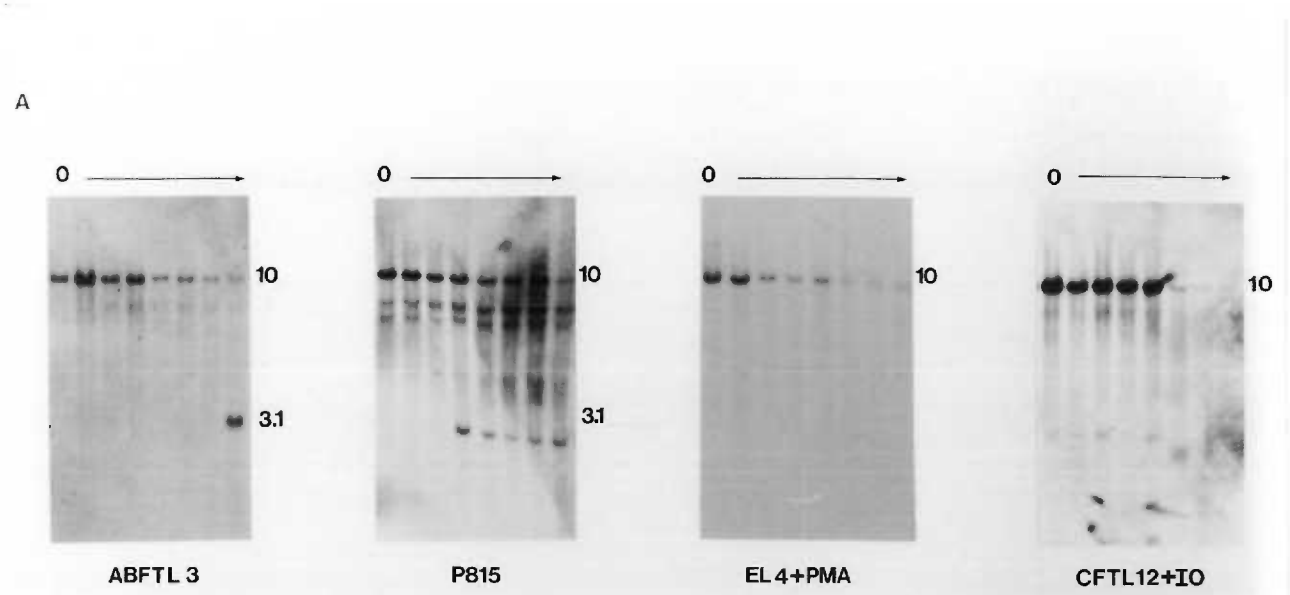


TIS Transcription initiation site

IL-4 exon

Hypersensitive site

Figure 3: DNAse I hypersensitive site analysis of Eco RI digested chromosomal DNA. A) Southern blot analyses of DNAse I treated DNA from unstimulated ABFTL 3 and P815 transformed mast cells and stimulated EL-4 T cells and CFTL 12 mast cells (increasing DNAse concentrations indicated by arrows above each blot). Cells were stimulated as described in figure 2. B) Localization of the HS sites on the murine IL-4 chromosomal map based on Eco RI digestion of DNAse I treated chromosomal DNA. The Hind III/ Rsa I fragment described in Figure 2 was used as a hybridization probe.


A
0
10
3.1
ABFTL 3
P815
EL4+PMA
CFTL12+IO

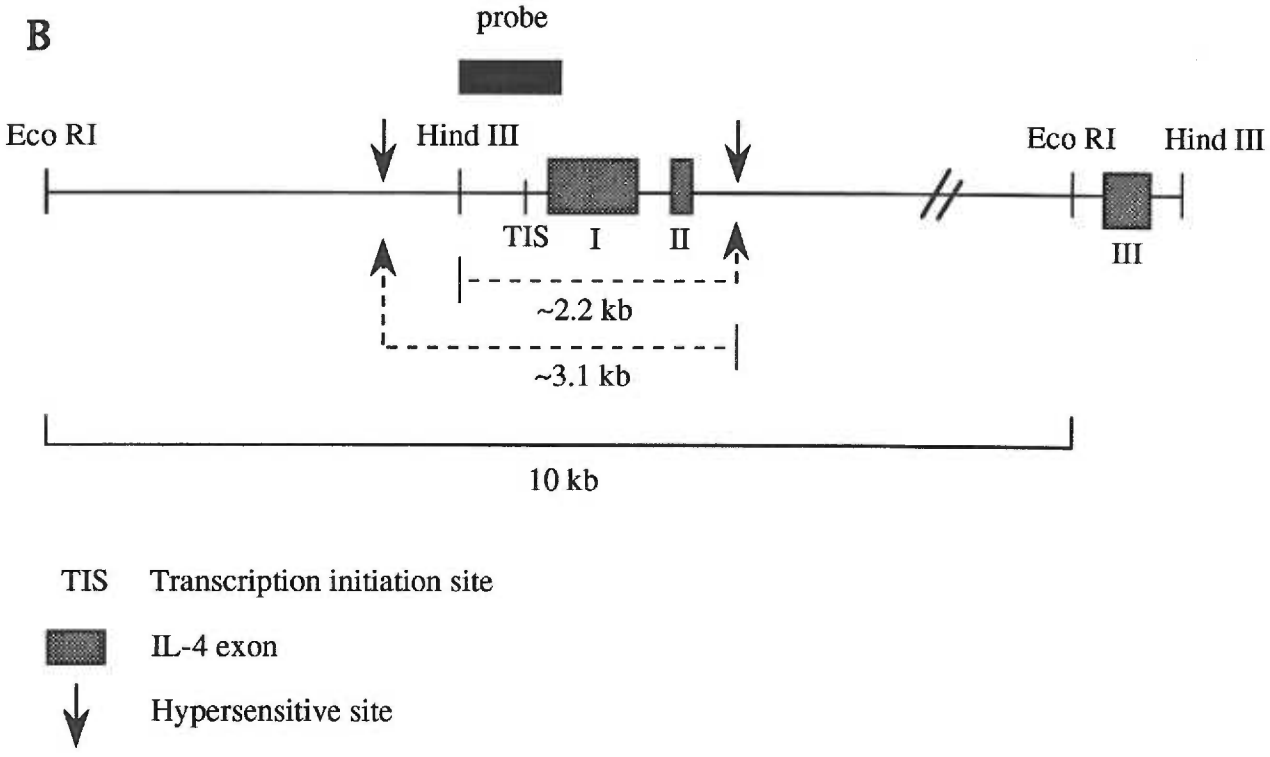

B
probe
Eco RI
Hind III
TIS
I
II
III
~2.2 kb
~3.1 kb
10 kb
TIS Transcription initiation site
IL-4 exon
Hypersensitive site

Figure 4: Partial murine IL-4 genomic map (previously reported by Otsuka *et al.* {27})and restriction fragments subcloned into pSV40-CAT reporter gene vector. Fragments were isolated using the indicated restriction enzyme sites and subcloned into either the Bam HI or Bgl II site. Filled boxes on linear map represent IL-4 exons. The fragment sizes indicated are approximate. SV40 derived sequences in the pSV40-CAT vector include poly A addition sites and splice sites. HS site designates the estimated position of the 3' DNAse I HS site with respect to the 670 bp Bgl II fragment.

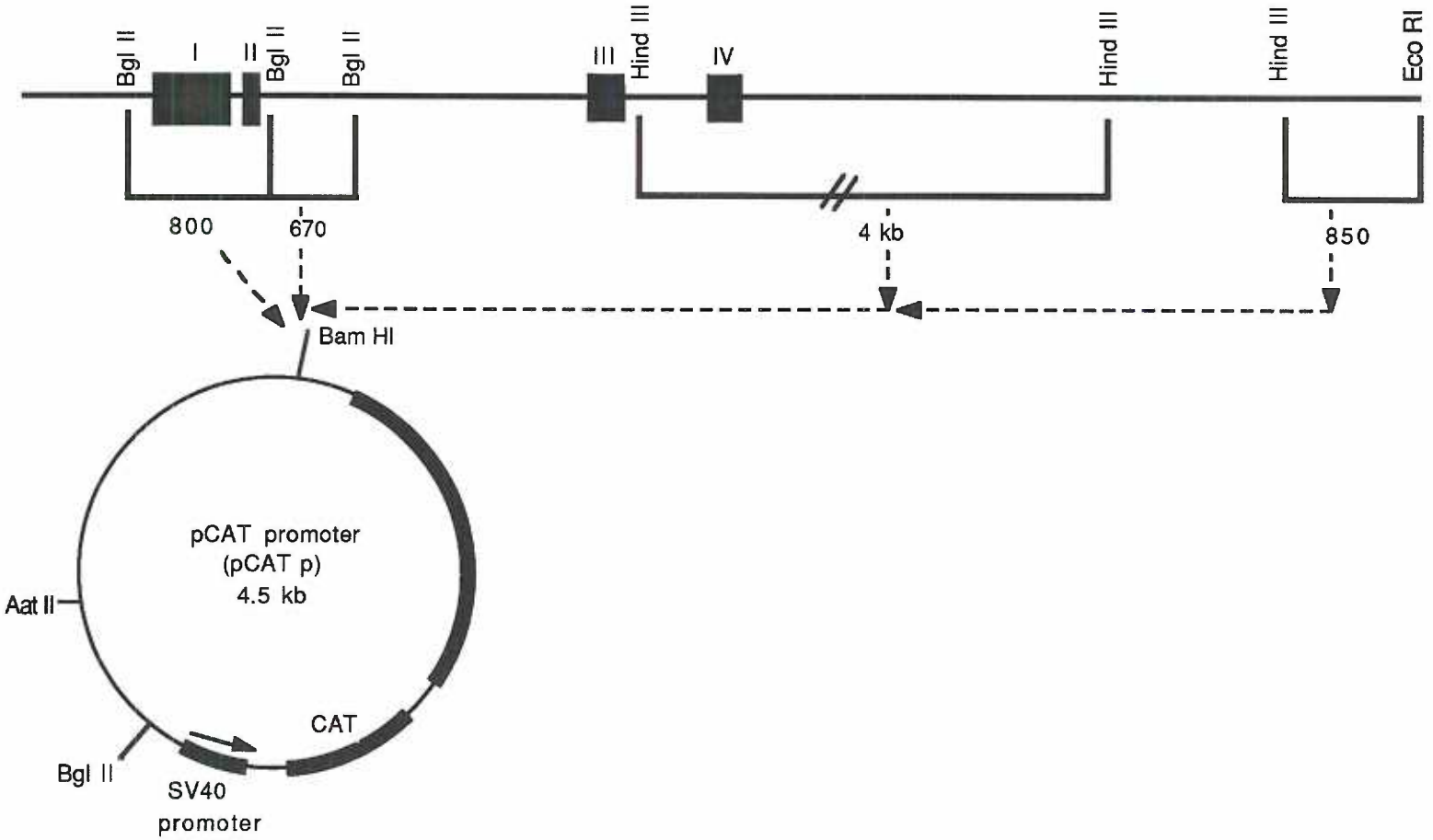

Bgl II
I
II
Bgl II
Bgl II
III
Hind III
IV
Hind III
Hind III
Eco RI
800
670
4 kb
850
Bam HI
pCAT promoter
(pCAT p)
4.5 kb
Aat II
CAT
Bgl II
SV40
promoter

Figure 5: A 670 bp Bgl II fragment from the second exon of the IL-4 gene enhances SV40 promoter driven CAT expression in three independently derived transformed mast cell lines. The 800 bp, 4 kb and 850 bp sequences cloned into pSV40-CAT (see Figure 4) did not show activity above pSV40-CAT promoter alone in these experiments. Conditions for electroporation and CAT assays are as described in Table I. *Fold increase in CAT activity relative to pSV40-CAT

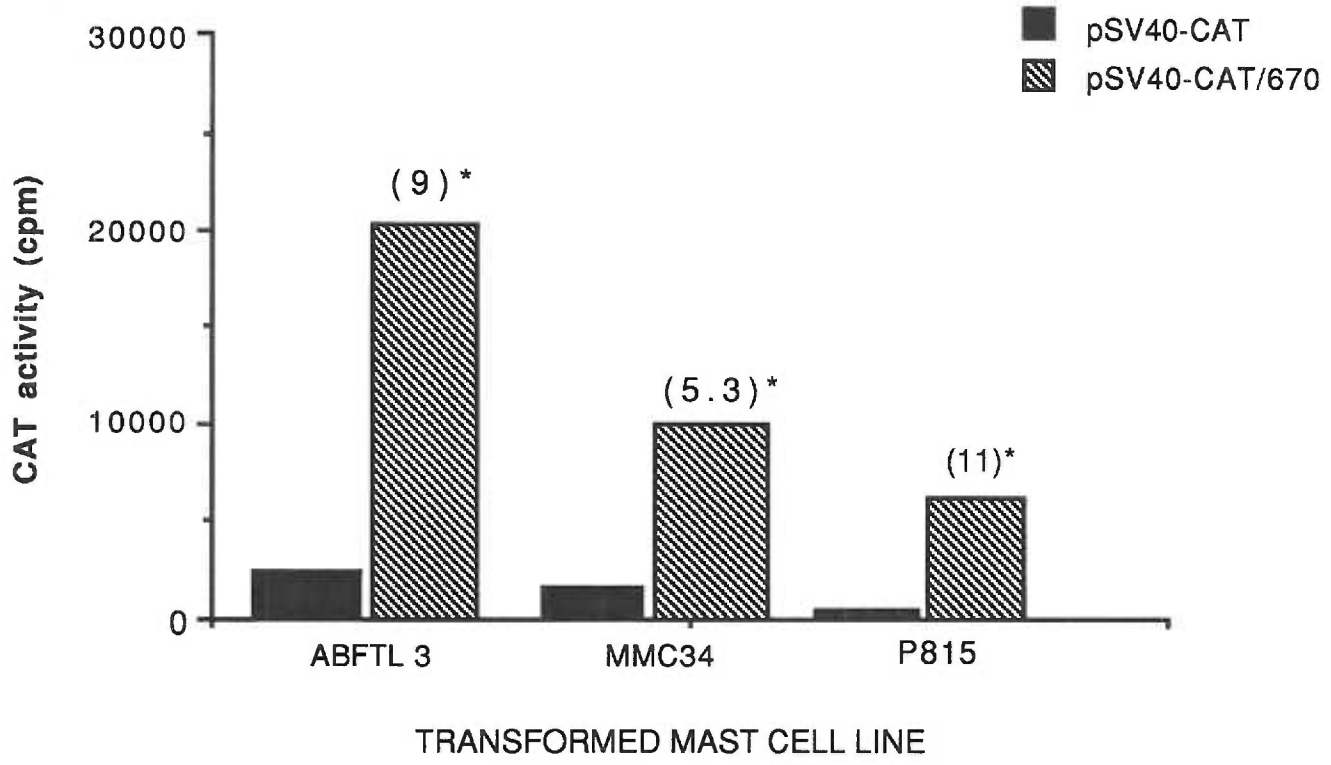

pSV40-CAT
pSV40-CAT/670
30000
20000
10000
0
CAT activity (cpm)
(9)*
(5.3)*
(11)*
ABFTL 3
MMC34
P815
TRANSFORMED MAST CELL LINE

Figure 6: The activity of the 670 bp intronic sequence is position and orientation independent. ABFTL 3 cells were transfected with constructs containing the 670 bp Bgl II fragment subcloned in both orientations into either the Bam HI or Bgl II site of pSV40-CAT (see Figure 4). Conditions for electroporation and CAT assays are as described in Table I. Proximal refers to constructs in which the sequence was cloned (in both orientations-ori 1 and 2) into the Bgl II site (immediately 5' of the SV40 promoter). Distal refers to constructs in which the 670 bp sequence was cloned into the Bam HI site of pSV40-CAT.

*Fold increase in CAT activity relative to pSV40-CAT

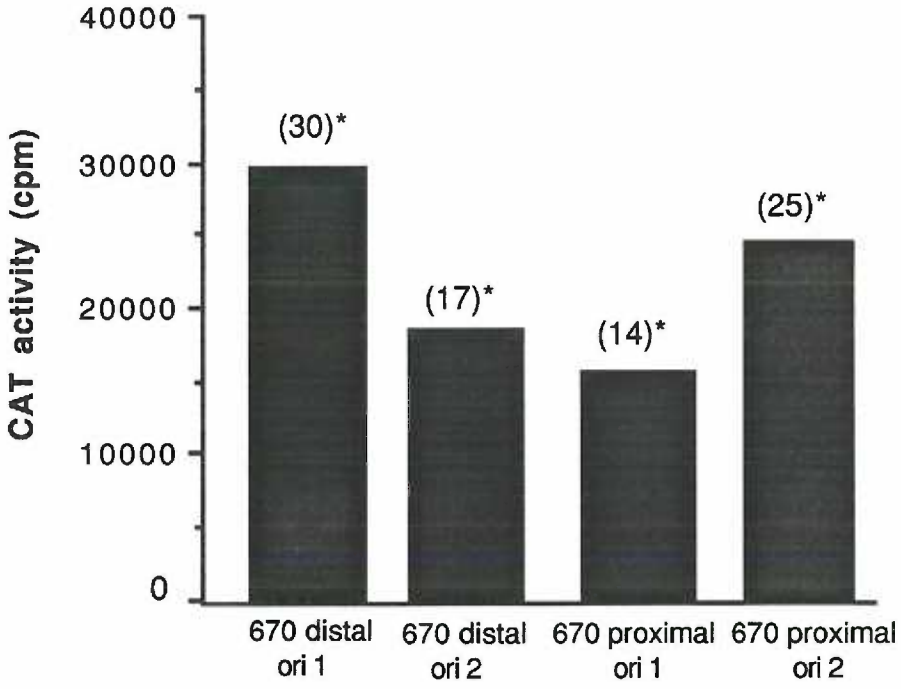

CAT activity (cpm)
40000
30000
20000
10000
0
(30)*
(17)*
(14)*
(25)*
670 distal ori 1
670 distal ori 2
670 proximal ori 1
670 proximal ori 2

Figure 7: The 670 bp Bgl II intronic sequence acts with both the IL-4 and SV40 promoter to enhance CAT expression in ABFTL 3 cells. Conditions for electroporation and CAT assays are as described in Table I. *Fold increase in CAT activity relative to pSV40 CAT (SV40 promoter) ** Fold increase in CAT activity relative to -797IL-4 pCAT (IL-4 promoter)

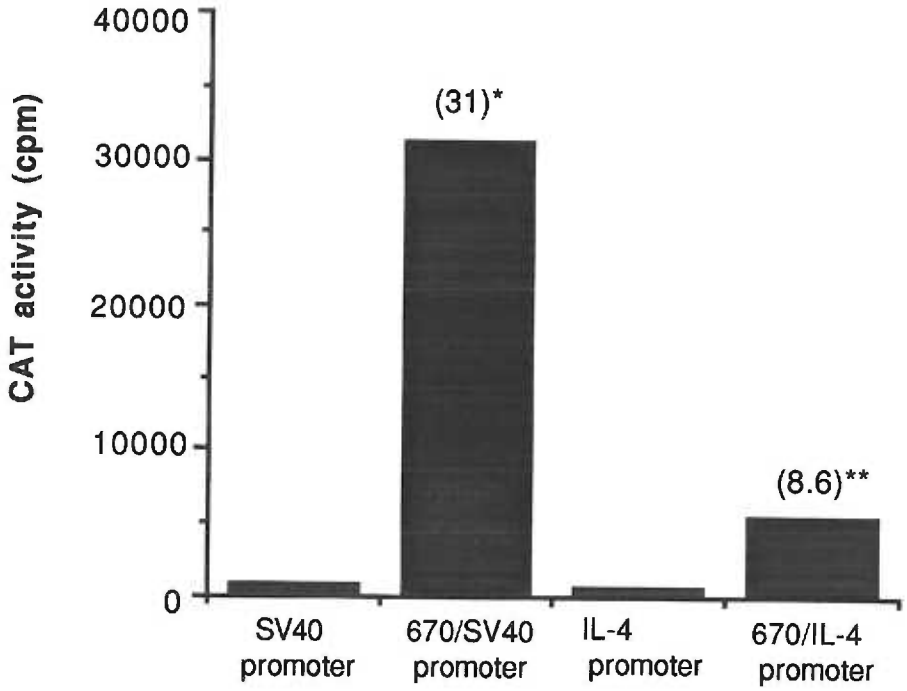

CAT activity (cpm)
40000
30000
20000
10000
0
(31)*
(8.6)**
SV40 promoter
670/SV40 promoter
IL-4 promoter
670/IL-4 promoter

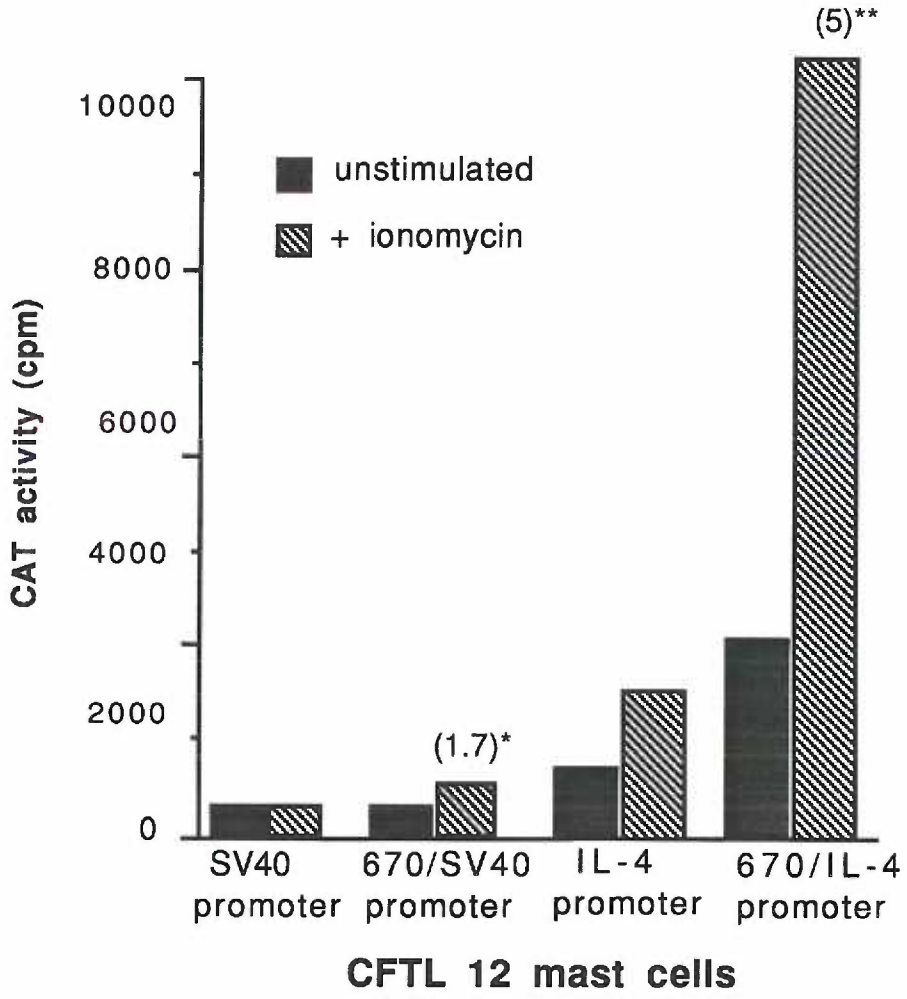
Figure 8: The intronic sequence acts with both the IL-4 and SV40 promoter to enhance CAT expression in CFTL 12 mast cells but is inactive in EL-4 T cells.

A. Analysis of enhancer activity in CFTL 12 mast cells. **B**. Analysis of enhancer activity in EL-4 T cells. Cells were transfected by electroporation. After 16 hours of culture, CFTL 12 cells were stimulated with 1 μM of ionomycin 8 hours before cell extracts were harvested. EL-4 cells were stimulated with 10 ng/ml of PMA for 24 hours before cell extracts were harvested. Unstimulated cell extracts were harvested 40 hours post-transfection.

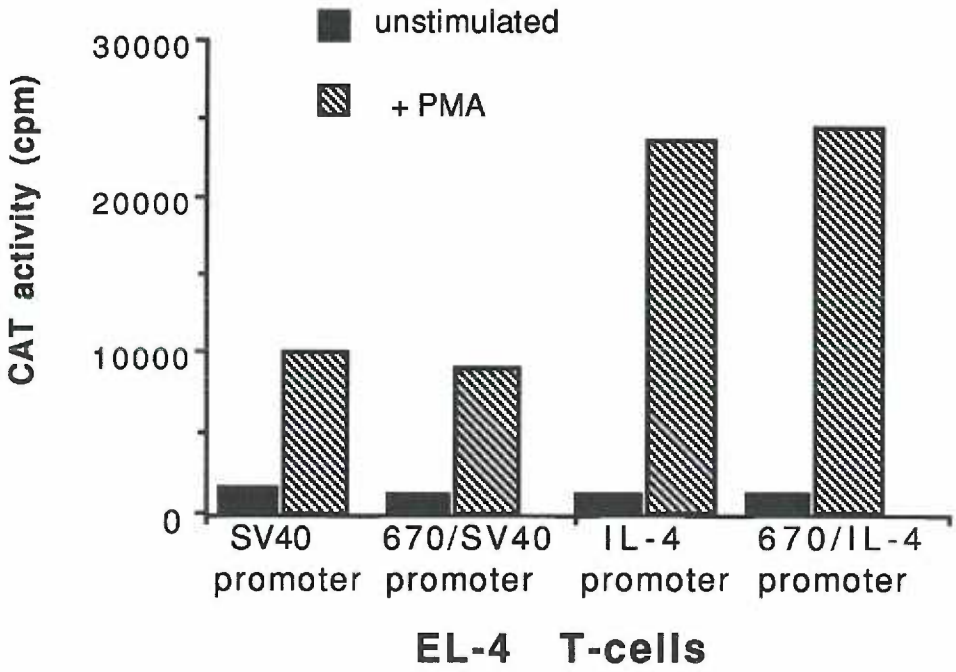
*Fold increase in CAT activity relative to pSV40-CAT (SV40 promoter)

** Fold increase in CAT activity relative to -797 IL-4 pCAT (IL-4 promoter)

A


(5)**
10000
8000
6000
4000
2000
0
unstimulated
+ ionomycin
CAT activity (cpm)
(1.7)*
SV40 promoter
670/SV40 promoter
IL-4 promoter
670/IL-4 promoter
CFTL 12 mast cells

B


30000
20000
10000
0
unstimulated
+ PMA
CAT activity (cpm)
SV40 promoter
670/SV40 promoter
IL-4 promoter
670/IL-4 promoter
EL-4 T-cells

II. Manuscript #2

PU.1 and GATA: Components of a Mast Cell-Specific IL-4 Intronic Enhancer

(transcription factors/cytokine/gene regulation)

Greg Henkel* and Melissa A. Brown[1+*#]

From the Departments of Microbiology/Immunology* and Medicine+
Oregon Health Sciences University, Portland, OR 97201

Contributed by William E. Paul
Classification: Immunology

Submitted to Proc. Natl. Acad. Sci. USA, Jan 15, 1994 (in press).

To whom reprint requests should be addressed.
Abbreviations: IL, interleukin; PMA, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay

Abstract

Interleukin 4, a critical immunoregulatory cytokine, is produced only by a subset of T lymphocytes and cells of the mast cell/basophil lineage. There are cell-specific differences in the regulatory elements that control IL-4 transcription in these two cell types. A 683 base pair Bgl II fragment, located within the second intron of the murine IL-4 gene, was previously shown to exhibit mast cell-specific enhancer activity. To define critical *cis* -acting elements that regulate this enhancer, a series of deletions from the 5' and 3' ends of the Bgl II fragment were generated. Their effect on enhancer activity was assessed in IL-4 producing mast cell lines in transient transfection assays. Two functionally independent subregions, E1 and E2, were defined in this analysis. Both are required for full enhancer activity. Sequences identical to previously defined DNA binding sites for SP1 and GATA are present within E1 and an ets binding site is located within E2. Mutation of the SP1 sites had no effect on enhancer function. However, alteration of either the GATA or ets site reduced enhancer activity by 50-60%. Proteins that associate with the IL-4 intronic GATA and ets sites were detected in mast cell nuclear extracts by mobility shift assays. Specific antibodies identified these factors as GATA-1 and GATA-2 and the ets family member, PU.1. GATA-1, GATA-2 and PU.1 exhibit cell-specific expression suggesting that these proteins play a critical role in the lineage-restricted activity of the IL-4 intronic enhancer in mast cells.

Introduction

The role of mast cells as the primary immune effector cells in IgE-dependent immediate hypersensitivity responses is well characterized. Upon activation, mast cells degranulate and release several different types of preformed and newly synthesized biological mediators that initiate a variety of events that lead to inflammation (1, 2). Within the last ten years, evidence has accumulated demonstrating that activated mast cells are also a major source of several cytokines, including IL-1, IL-3, IL-4, IL-6 and TNF-α (3-7). These findings indicate an additional role for mast cells in processes such as late phase inflammatory responses, angiogenesis, and wound healing (5, 8). Mast cells are activated by either specific multivalent antigen-IgE complexes bound to the cell surface high affinity Fcε receptor (FcεRI) or through IgE-independent interactions involving neuropeptides, anaphylatoxins or cytokines (2, 9). Some of these same stimuli have also been shown to initiate cytokine production. (3, 4, 10).

Among the mast cell-derived cytokines, Interleukin-4 (IL-4) is of particular interest. IL-4 is an immunoregulatory cytokine that exhibits diverse effects on a wide variety of cells including an ability to regulate the growth and differentiation of T and B lymphocytes (11). IL-4 has a unique function in regulating IgE production by B cells and is a mast cell growth factor (11-13). These properties suggest that IL-4 has autocrine activity and plays a major role in regulating mast cell function. In addition, mast cell-derived IL-4 may have distinct effects on many immune and physiologic responses when compared to IL-4 produced by T cells; mast cells are concentrated in different anatomical sites and are widely distributed in vascularized tissues in close proximity to distinct target cells (2, 8).

Several recent reports have described *cis*-acting elements that regulate the transcription of IL-4 in T cells (14-20). However, little is known about the nuclear events that control cytokine expression in mast cells. Previous studies by our laboratory have utilized IL-3-dependent mast cell lines derived from fetal liver cells to study events that regulate the physiological activation-dependent expression of IL-4 (21). In addition, a number of growth factor-independent, transformed mast cell lines express high levels of IL-4 mRNA constitutively (22). We demonstrated that these mast cells exhibit a unique DNAse I hypersensitive site within the second intron of the murine IL-4 gene. This site defines a regulatory element located on a Bgl II restriction fragment that has many of the characteristics of a prototypic enhancer sequence. Its activity in transient transfection/reporter gene assays is independent of orientation and position and it acts with both the IL-4 promoter and a heterologous SV40 promoter. Notably, this enhancer functions in both transformed mast cells and the factor-dependent mast cell lines but not in IL-4 producing EL-4 T cells or other cell types that do not express IL-4. Therefore, the activity of this enhancer appears to be mast cell-specific. In this study we have identified critical sequences that regulate this enhancer activity. These *cis* -acting elements specifically interact with proteins belonging to the GATA and ets family of transcription factors. The restricted expression of these factors in mast cells, but not T cells, indicates that they play a role in the mast cell-specific activity of the IL-4 intronic enhancer.

Materials and Methods

Cell Lines. ABFTL-3 is a murine transformed mast cell line derived from *in vitro* Abelson MuLV infection of IL-3 dependent mast cells (23). MMC34 is a murine transformed mast cell line isolated after *in vivo* Abelson MuLV (24). P815 is a transformed mast cell line isolated from a methylcholanthrene-induced mastocytoma (25). CFTL-12 is a factor-dependent, non-transformed mast cell derived from fetal liver (23). EL-4 T-cells (26), GA15 T-cell hybridoma (27), WEHI 231 B-cells (28) and CH12 LX 4550 B-lymphoma (29) have been previously described. All cells were passaged twice weekly in 1640 RPMI supplemented with 10% bovine calf serum, penicillin/streptomycin and 50 μM 2-mercaptoethanol. CFTL 12 mast cells were also supplemented with 25% IL-3 containing conditioned media from WEHI 3 cells (30). To induce IL-4 expression, CFTL 12 mast cells were treated with 0.75 μM ionomycin (Cal Biochem, San Diego, CA.), EL-4 T-cells were treated with 20 ng/ml PMA (Sigma Chemical Co., St. Louis, MO.) and GA15 were incubated with immobilized 2C11, an anti-CD3 specific monoclonal antibody (31). Flasks were coated with 1 μg/ml 2C11 overnight at 4°C before the addition of the cells.

Reporter Gene Constructs. Details of the derivation of 683/SV40 CAT have been previously described (21). [Note: in the original description, this construct was called 670/SV40 CAT. Upon sequencing the enhancer element, it was found to contain 683 base pairs and is now referred to as 683/SV40 CAT]. To generate deletions from either the 5' or 3' end of the 683 bp fragment, two forms of the 683/SV40 CAT vector, with the intronic sequence cloned in each orientation, was linearized at one end of the enhancer element with Xba I and treated for various times with Bal 31

exonuclease (Pharmacia, Piscataway, N.J.). Bal 31-treated samples were pooled, the ends polished with T4 polymerase and the vector was religated with T4 ligase. The extent of deletions was determined by DNA sequencing of double-stranded DNA plasmid templates using the dideoxy chain termination method (32). The numbering of nucleotides within the 683 base pair fragment is based on designation of the first base of the 5' Bgl II recognition site as number 1.

pF202 was constructed by using PCR primers, including IL-4 intronic sequences from 353-372 and 554-535, to generate a fragment containing sequences from 353 to 554. These primers have an additional 10 nucleotides at the 5' end that contain a Pst I site. The PCR product was digested with Pst I and subcloned into the Pst I site of pCAT promoter. The sequence was verified as described above.

Site-directed Mutagenesis. Mutations within the SP1, GATA and ets consensus binding sites, were introduced into the IL-4 intronic sequence containing base pairs 255-683 (demonstrates full enhancer activity relative to the 683 base pair fragment) using the oligonucleotide-directed *in vitro* mutagenesis system version 2.1 (Amersham, Arlington Heights, Ill.). All mutations were verified by sequencing. The mutant forms of the oligonucleotides used in the mutagenesis reactions were synthesized using an Applied Bioscience Oligonucleotide Synthesizer and are listed below.

IL-4 SP1 bp. #410-387

wild-type	5' CAAATGGAGGGGCGGGGGGCAGGG 3'
mutant (SP1-M_A)	5' CAAATGGAGCAGCTGGGGGCAGGG 3'

IL-4 SP1 bp. #431-409

wild-type	5' CACATCTGTCCCCTCCCACTACA 3'

mutant (SP1-M_B) 5' CACATCTGTCGGATCCCACTACA 3'

IL-4 GATA bp. #454-430

wild-type 5' GAGAAAATGCACTATCAGCCTCTCA 3'

mutant (GATA-M1) 5' GAGAAAATGCATCTAGAGCCTCTCA 3'

IL-4 ets bp. #520-496

wild-type 5' CCATGAAAACAGGAACTGAAATGCA 3'

mutant (ets-M1) 5' CCATGAAAACATTCACTGAAATGCA 3'

Transfection and CAT assays. 10^7 ABFTL 3 cells were transfected by electroporation with 6.73 pmoles (20-23 μg) of the various CAT reporter plasmids. Cells were harvested 48 hours later and equal amounts of cell extracts were assayed for CAT activity using a scintillation diffusion assay as previously described (21).

Electrophoretic mobility shift assays. Nuclear extracts were made using a method described by Fiering *et al.* (33), but were not passed over a P6DG column. Extracts from stimulated, factor-dependent mast cells were made 30-60 minutes after treatment with 0.75 μM ionomycin. Extracts from stimulated EL-4 T-cells or GA15 were isolated after a 12 hour induction with 20 ng/ml PMA or with immobilized 1 μg/ml 2C11 antibody, respectively. Total protein in each extract was quantitated using the Bio Rad protein assay kit (Bio Rad, Richmond, CA) .

The binding reactions included 5 μg of nuclear extract and 0.1 ng ^{32}P-labeled probe, in a buffer containing 10 mM Hepes-KOH, pH 7.8, 50-75 mM KCl, 0.5 mM $MgCl_2$, 2 mM DTT, 0.1% Triton X-100, 5% glycerol and 0.3-1.0 μg of poly [dI.dC]. Reactions were incubated at room temperature for 30-60 minutes and electrophoresed on a 5% polyacrylamide

gel in a Tris-glycine buffer (25 mM Tris, 190 mM glycine and 1 mM EDTA). For the mobility shift assays using antibodies to identify the proteins associated with the DNA probes, 1 µl of antiserum or preimmune serum was added to the binding reaction prior to the addition of the probe and allowed to incubate for 15 minutes at room temperature. Both GATA-1 monoclonal antibody and GATA-2 antiserum were the kind gift of Dr. Stuart Orkin, Children's Hospital, Boston Mass. The PU.1-9794 antiserum (34) was a generous gift from Dr. David Kabat, Oregon Health Sciences University, and the PU.1 peptide antiserum, 1297 (35), was generously provided by Dr. Richard Maki, La Jolla Cancer Research Foundation. The IL-4 oligonucleotides containing the GATA and ets sites used in these experiments are listed below. The sites identical to previously described consensus sites are in bold type.

IL-4 GATA bp. #422-450

5' GACAGATGTGACAGGCTGATAGTGCATTT 3'

IL-4 ets bp. #496-520

5' TGCATTTCAGTTCCTGTTTTCATGG 3'

Results

Two distinct subregions contribute to IL-4 enhancer activity. We previously identified a 683 base pair Bgl II fragment within the second intron of the IL-4 gene that exhibits mast cell-specific enhancer activity (21). To define the precise *cis*-acting elements that regulate this enhancer, deletions from both ends of the Bgl II fragment were generated using Bal 31 exonuclease. The effects of these deletions on the enhancement of SV40 promoter-driven CAT gene expression in ABFTL-3 transformed mast cells was determined. These cells produce high levels of IL-4 mRNA constitutively. The results of these experiments are shown in Figures 1A and 1B. Three deletion constructs, Δp1 and Δp2, in which sequences from 1 to 345 are deleted, and Δp5, containing a deletion from 549 to 683 retain full activity indicating that enhancer activity is localized between 346 and 548. (Note: the numbering of the coordinates is based on designation of the first base of the 5' Bgl II site as #1). A PCR generated sequence from 353 to 554 enhanced CAT gene expression at levels equivalent to the entire 683 bp fragment (Figure 1C), confirming these results.

The partial activity of constructs containing further deletions suggests that the enhancer is comprised of at least two elements that can act independently to enhance transcription. There is a 50 to 60% decrease in activity when comparing the response of the 5' deletion constructs Δp2 and Δp3. These results indicate the sequences between 346 and 444 are critical for full enhancer activity. This region has been designated E1 (Figure 1A). The residual activity of Δp4 (40 to 50% of 683/SV40 CAT) defines a second important region that is located between 496 and 548. A comparison of the activity of the 3' deletion constructs, Δp5 and Δp6 in which there is a 50 to

60 % decrease in activity confirms the presence of this second region, designated E2 (Figure 1B). Furthermore, the contribution of E1 was confirmed by the decrease in activity of Δp7 when compared to Δp6.

Sequences identical to binding sites for previously described transcription factors are located within the E1 and E2 subregions. A partial sequence spanning the entire functional region of the enhancer is shown in Figure 2. Analysis of this sequence revealed that there are several sequences that demonstrate homology to previously defined *cis* -acting regulatory elements. However, four sites are identical to binding sites for previously described transcription factors. These sites are located precisely within the two functional subregions, suggesting a potential role in IL-4 enhancer function. Two SP1 binding sites [392-402 and 415-422] (36) and a GATA binding site [438-443] (37) were identified within E1 (Figure 2). An ets binding site (38) is located within the E2 region at position 503-510.

The IL-4 GATA and ets-like binding sites are critical for enhancer activity. To assess the possible contribution of SP-1, GATA and ets sites in IL-4 enhancer activity, single or multiple site-directed mutations which disrupt each of the core binding sites were introduced into Δp1 (255 to 683), a construct that exhibits full enhancer activity (See Figure 1A). The effect of these mutations on CAT gene expression in mast cells is shown in Figure 3. Mutation of a single SP-1 site (not shown) or both SP-1 sites together (dmSP-1) has no effect on enhancer function, whereas mutation of either the GATA (mt-GATA) or ets-like site (mt-ets) reduces enhancer activity by 50-60%. These results indicate that both the GATA and ets sites contribute to enhancer activity. Furthermore, they confirm the previous

conclusions that discrete elements within E1 and E2 have independent transactivation capability.

It is notable that mutations in both the GATA and ets sites (mt-ets/GATA) reduced enhancer activity even further relative to the constructs with single mutations, but not to the level of the pCAT promoter alone. This data suggests that an as yet unidentified element(s) is interacting with both the GATA and ets elements to regulate enhancer function and is required for full enhancer activity. DNAse I footprinting experiments demonstrate that sequences between 466 and 487 are protected (G. Henkel, unpublished observation). We are currently examining the potential role of this region in enhancer function.

The GATA and ets sites are the target of specific DNA-protein interactions. The functional data indicates that both the GATA and ets *cis* elements are necessary for enhancer function and strongly suggests that transcription factors belonging to the GATA and ets family interact with these sequences. To verify that specific proteins, present in ABFTL-3 mast cells, are associated with these sites, electrophoretic mobility shift assays (EMSAs) were performed using labeled oligonucleotide probes containing either the IL-4 GATA or ets binding site (Figure 4). Specific protein/DNA interactions are detected with both probes (lanes 1 and 5). Two complexes form with the GATA probe (designated A and B). A single protein-DNA complex is observed with the ets probe. The specificity of these interactions was confirmed in EMSA experiments in which an excess of unlabeled competitor DNA was added to the binding reactions. The detection of both GATA complexes is inhibited by the addition of 100-fold excess unlabeled GATA probe (lane 2), but not with the same amount of a GATA probe containing a mutation in the core binding site (lane 3) or the unrelated IL-4

ets site probe (lane 4). Similarly, detection of the ets complex is abolished by an excess of IL-4 ets site DNA but not by a mutant ets probe or the IL-4 GATA probe (lanes 6, 7 and 8 respectively). The inability of the mutant GATA and ets site probes to compete correlates with the loss of enhancer activity observed with the Δp1 constructs containing mutations in those sites.

The mast cell-specific activity of the intronic enhancer correlates with the cell-restricted distribution of the factors that bind to the GATA and ets sites. IL-4 intronic enhancer function is restricted to mast cells that express the IL-4 gene (21). It is possible that differential binding of transcription factors to the GATA and/or ets sites could explain the cell type specificity of this enhancer. To explore this possibility, nuclear extracts from several IL-4 producing T, B and mast cell lines and a non-IL-4 producing B cell line were examined by EMSA for binding activity to IL-4 GATA and ets site probes. As shown in Figure 5A, GATA binding activity is present in all of the cell lines, although there are marked differences in the number and mobility of the complexes observed. The specificity of these interactions was confirmed in competition experiments as described above (data not shown). With the exception of P815 (lane 2), extracts from all the mast cell lines form two complexes identical to the those observed with ABFTL 3 extracts (lanes 1, 3-5). P815 extracts form only the complex of lower mobility. Extracts from T (lanes 6-9) and B cell lines (lanes 10, 11) also form specific IL-4 GATA site complexes but they migrate at mobilities distinct from the mast cell complexes. In addition, B cell extracts contain a significantly reduced amount of GATA binding activity compared to the other cell types examined.

Cell-specific differences in binding activity to the IL-4 ets site probe were also observed (Figure 5B). All mast cell extracts form a single specific complex of the same mobility as the ABFTL 3 DNA-protein interaction. However, no binding activity is observed in either unstimulated or stimulated T cell extracts. This is noteworthy because T cells express several ets family members (39). B cells, however, contain specific binding activity that migrates with the same mobility as the mast cell ets complex.

GATA-1, GATA-2 and PU.1 specific antibodies react with proteins that bind to the GATA and ets sites within the IL-4 intronic enhancer. Proteins that bind GATA motifs belong to a family of factors that share a highly conserved DNA binding domain (40). GATA-1 and GATA-2 are primarily expressed in erythrocytes, megakaryocytes and mast cells, but not T cells (40). Thus, it was of interest to determine whether GATA-1 and GATA-2 are present in the IL-4 producing mast cell lines that we have studied and bind to the IL-4 GATA site. Antibodies that specifically react with each factor were added to standard EMSA binding reactions containing ABFTL-3 nuclear extracts. As shown in Figure 6, a GATA-1 specific monoclonal antibody blocks complex A formation (lane 2) and has no effect on complex B. GATA-2 antiserum interferes with complex B formation (lane 3) but has no effect on GATA-1 complex formation. Pre-immune rabbit serum as well as an ets family specific antiserum were used as controls and did not affect the formation of either complex A or B (lanes 4 and 5). The GATA-specific antibodies do not react with proteins associated with the EL-4 T cell GATA complexes (data not shown).

There is also a large family of ets site binding factors that are related by virtue of a common DNA binding domain (41). These family members recognize small differences in the ets binding sites within the regulatory regions of several genes (38). Two observations indicated that the ets family member, PU.1, may be associated with the IL-4 ets site. The intronic ets-like site shares 10 of 11 base pairs of identity with the SV40 PU.1 binding site (42). Furthermore, we observed that mast cells and B cells, but not T cells, exhibit IL-4 ets site binding activity. This cell-specific expression resembles that which has been described for PU.1 (43-45). To determine if PU.1 is binding to the IL-4 ets sequence, mobility shift assays were performed with the IL-4 ets site probe using ABFTL 3 nuclear extracts in the presence of two different PU.1 specific antibodies (Figure 7). An extract from WEHI 231 cells, a B cell line previously shown to express PU.1 (46), was used as a positive control. PU.1-9794 antiserum, from animals immunized with the whole PU.1 protein (34), was able to inhibit IL-4 ets DNA complex formation (lanes 2 and 7). A PU.1 peptide specific antiserum reactive with a region outside the highly related DNA binding domain (35) also inhibits complex formation with the IL-4 ets probe (lanes 3 and 8). Neither preimmune serum or anti-GATA-1 has an effect on the ets complex (lanes 4, 5 and 9, 10).

Discussion

IL-4 is produced by a subset of T cells and cells of the mast cell/basophil lineage (11). These cells produce high levels of IL-4 mRNA and protein when activated (3, 4, 11, 47). However, the cell surface receptors that trigger inducible expression of IL-4 by each cell type are distinct (11). Thus, the regulatory mechanisms that control IL-4 expression within these cell types are likely to be different. The discovery of a sequence within the second intron of the murine IL-4 gene that exhibits mast cell-specific enhancer activity supports this hypothesis (21). In this study we have characterized the *cis* and *trans*-acting elements that contribute to the cell-restricted activity of this enhancer. Two functionally independent subregions, designated E1 and E2, were identified by analysis of the ability of serial 5' and 3' end deletions of the 683 base pair intronic fragment to enhance CAT reporter gene expression. Within these subregions, there are potential binding sites for previously described transcription factors. Although alteration of two conserved SP-1 binding sites within E1 does not effect CAT activity, mutations within either the core recognition sequence of a GATA binding site in the E1 subregion or an ets binding site in the E2 subregion results in a 50-60% loss of enhancer activity. These data indicate that both the GATA and ets-like *cis*-acting elements contribute to enhancer activity. In addition, specific DNA binding proteins interact with both these sites in *in vitro* DNA-protein interaction assays. Antibodies identified these proteins as GATA-1 and GATA-2 as well as the ets family member, PU.1. Experiments to confirm an *in vivo* role for these proteins in regulating enhancer activity are currently underway.

The GATA proteins, present in ABFTL 3 cells, exhibit highly restricted, cell-specific expression indicating that they may regulate the mast cell-specific activity of this enhancer. Our analysis of IL-4 GATA-site nuclear binding activity in other cell lines supports this conclusion. Mast cell lines in which the intronic enhancer is active express GATA-1 and/or GATA-2. These same transcription factors regulate the mast cell-specific expression of the carboxypeptidase A gene (MC-CPA) (48). In contrast, although T and B cells contain specific GATA binding activity, it forms complexes of distinct mobility compared to the mast cell complexes in EMSAs and fails to react with GATA-1 and GATA-2 specific antibodies. This result is consistent with other studies showing GATA-1 and GATA-2 are expressed in cells of the mast, erythroid and megakaryocyte lineage but not T or B lymphocytes (40).

The relative contribution of GATA-1 and GATA-2 to enhancer activity also remains to be determined. The intronic enhancer is active in P815 cells, another transformed mast cell line that expresses high levels of IL-4 mRNA constitutively (21), yet nuclear extracts from these cells form only a single GATA protein-DNA complex (complex B-Figure 5A). We have confirmed this complex contains GATA-2 (G. Henkel, data not shown), consistent with a previous report demonstrating that P815 cells express GATA-2, but not GATA-1, binding activity (48). Therefore, GATA-2 appears to be sufficient for mediating at least partial enhancer activity. However, a contribution by GATA-1, present in the other mast cell lines, cannot be excluded. In studies of GATA-mediated MC-CPA promoter activity in P815 cells, stable transfection and expression of a GATA-1 cDNA resulted in an increase in the ability of the MC-CPA promoter to drive the expression of a human growth hormone reporter gene in transient transfection experiments (48).

It is likely that the T cell lines we examined express another member of the GATA family, GATA-3. This transcription factor is expressed primarily in T cells, including the EL-4 cell line, and non-hematopoietic cells (40, 49). GATA-3 binds to the WGATAR consensus site with a high affinity and regulates the transcription of several T-cell receptor genes (50). Thus, it is curious that although T cells contain specific GATA binding activity, the enhancer fails to function (21). We speculate that there may be differences in the ability of GATA-3 to bind to the IL-4 intronic GATA site *in vivo* or there are additional factors, absent in T cells, that are required for GATA-mediated activity. Alternatively, GATA-1, GATA-2 and GATA-3 demonstrate divergent amino acid sequences outside their DNA binding domains (40), and these may result in differences in trans-activation capabilities.

Based on its presence in IL-4 producing mast cells but not T cells, another cell-restricted transcription factor, PU.1, also appears to contribute to the cell-specific activity of the intronic enhancer. Previously defined as a macrophage and B cell-specific transcription factor (43), this ets family member is a regulator of the myeloid-specific CD11b promoter and the B cell-specific J chain gene promoter (35, 51). Two recent reports have also demonstrated that PU.1 is expressed by mast cells (44, 45). It is notable that although T lymphocytes express other ets family members (39), we are unable to detect any protein binding to the IL-4 intronic ets site in T cells. This result indicates that this site is highly specific for PU.1 and suggests that the IL-4 ets sequence itself plays a role in the cell-restricted activity of this enhancer. This observation is further evidence that in addition to differential expression of transcription factors, microheterogeneity within

transcription factor binding sites plays a major role in regulating DNA-protein interactions (38, 52, 53).

Both the IL-4 GATA and ets sites must be intact for full enhancer activity suggesting cooperation between these sites and their associated proteins. An example of cooperation between GATA and ets *cis*-acting elements has also been described for the promoter of the human glycoprotein IIB gene (54). However, additional regions between nucleotides 346 and 548 are also likely to contribute to enhancer function. Mutation of both the GATA and ets sites together does not eliminate all enhancer activity (Figure 3). DNAse I footprint analysis demonstrated a candidate region between E1 and E2 (bp 440 to 470) that specifically interacts with proteins (G. Henkel, unpublished observation). The potential contribution of this region and others is currently being examined.

Questions regarding the physiologic role of the intronic enhancer remain unanswered. Although first defined in transformed mast cell lines, the enhancer functions, albeit at lower levels, in unstimulated, non-transformed mast cells (21). This activity correlates with the protein/DNA binding data that demonstrates GATA and PU.1 factors are present in unstimulated cells. It was recently demonstrated that mast cells store IL-4 and other cytokines in a preformed state (7, 55). Therefore, it is possible that this enhancer region plays a role in regulating the normal basal expression of IL-4 mRNA needed to translate protein for storage in the mast cell secretory vesicles. In transformed mast cells, high constitutive activity of the enhancer correlates with high basal expression of IL-4 mRNA. Thus, aberrant activity of a normal mast cell regulatory element may lead to the deregulated expression in these cells. We are unable to detect any differences between GATA and ets site binding proteins from non-transformed versus transformed cells.

However, there may be unique protein interactions or post-translational modification of proteins associated with these or other sites, not detected in EMSAs, that upregulate the activity of the enhancer in transformed cells.

The contribution of an analogous enhancer to IL-4 production in human mast cells is also unknown. Comparison of the murine intronic enhancer sequence with the equivalent region in the human IL-4 gene (56) demonstrates there is complete identity within the GATA and ets core binding sites as well as the closely flanking sequences (G. Henkel, unpublished observation). This conservation suggests the possibility that the same elements regulate IL-4 production in human mast cells.

In summary, the IL-4 intronic enhancer is regulated by multiple *cis*-acting elements that function independently. At least two mechanisms appear to contribute to its mast cell-restricted activity: 1) differential expression of transcription factors in mast versus T cells and 2) microheterogeneity in the ets DNA binding site that limits binding to specific members of the ets protein family. It will be important to determine whether or not this enhancer plays a role in regulating IL-4 production in human cells, exhibits the same mast cell-restricted activity and is a possible target of dysregulation in disease processes such as allergy and asthma in which IL-4 producing FcεRI$^+$ cells are likely involved (6, 7, 47).

Acknowledgments

We thank Drs. David Kabat, Stuart Orkin and Richard Maki for providing the antibodies used in these studies, Shane Fanning and Jenny Dang for technical assistance, Dr. Deborah Weiss, David Tara and John Hural for helpful discussions and Drs. Miles Wilkinson, David Henner and John Ansel for critical review of the manuscript.

These studies were supported by a grant from the Allergy and Asthma Foundation of America and National Institutes of Health grant AI34040.

References

1. Czarnetzki, B. M. (1987) *Semin. Dermatol.* **6,** 272-282.

2. Enerback, L. & Norrby, K. (1989) *Current Topics in Pathology* **79,** 169-204.

3. Burd, P. R., Rogers, H. W., Gordon, J. R., Martin, C. A., Sundararajan, J., Wilson, S. D., Dvorak, A. M., Galli, S. J. & Dorf, M. E. (1989) *J. Exp. Med.* **170**, 245-257.

4. Plaut, M., Pierce, J. H., Watson, C. J., Hanley-Hyde, J., Nordan, R. P. & Paul, W. E. (1989) *Nature* **339**, 64-67.

5. Gordon, J. R., Burd, P. R. & Galli, S. J. (1990) *Imm. Today* **11**, 458-464.

6. Bradding, P., Feather, I. H., Howarth, P. H., Mueller, R., Roberts, J. A., Britten, K., Bews, J. P. A., Hunt, T. C., Okayama, Y., Heusser, C. H., Bullock, G. R., Church, M. K. & Holgate, S. T. (1992) *J. Exp. Med.* **176**, 1381-1386.

7. Bradding, P., Feather, I. H., Wilson, S., Bardin, P. G., Heusser, C. H., Holgate, S. T. & Howarth, P. H. (1993) *J. Immunol.* **151**, 3853-3865.

8. Galli, S. J. (1993) *N. Eng. J. Med.* **328**, 257-265.

9. Church, M. K., Lowman, M. A., Robinson, C., Holgate, S. T. & Benyon, R. C. (1989) *Int. Arch Allergy Appl .Immunol.* **88,** 70-78.

10. Ansel, J. C., Brown, J. R., Payan, D. G. & Brown, M. A. (1993) *J. Immunol.* **150**, 4478-4485.

11. Paul, W. E. (1991) *Blood* **77**, 1859-1870.

12. Coffman, R. L., Ohara, J., Bond, M. W., Carty, J., Zlotnik, A. & Paul, W. E. (1986) *J. Immunol.* **136**, 4538-4541.

13. Mosmann, T. R., Bond, M. W., Coffman, R. L., Ohara, J. & Paul, W. E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5654-5658.

14. Abe, E., Malefyt, R. D., Matsuda, I., Arai, K. & Arai, N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 2864-2868.

15. Li-Weber, M., Eder, A., Krafft-Czepa, H. & Krammer, P. H. (1992) *J. Immunol.* **148**, 1913-1918.

16. Li-Weber, M., Krafft, H. & Krammer, P. H. (1993) *J. Immunol.* **151**, 1371-1382.

17. Todd, M. D., Grusby, M. J., Lederer, J. A., Lacy, E., Lichtman, A. H. & Glimcher, L. H. (1993) *J. Exp. Med.* **177**, 1663-1674.

18. Szabo, S. J., Gold, J. S., Murphy, T. L. & Murphy, K. M. (1993) *Mol. Cell. Biol.* **13**, 4793-4805.

19. Tara, D., Weiss, D. L. & Brown, M. A. (1993) *J. Immunol.* **151**, 3617-3626.

20. Bruhn, K. W., Nelms, K., Boulay, J. L., Paul, W. E. & Lenardo, M. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9707-9711.

21. Henkel, G., Weiss, D. L., McCoy, R., Deloughery, T., Tara, D. & Brown, M. A. (1992) *J. Immunol.* **149**, 3239-3246.

22. Brown, M. A., Pierce, J. H., Watson, C. J., Falco, J., Ihle, J. N. & Paul, W. E. (1987) *Cell* **50**, 809-818.

23. Pierce, J. H., Di Fiore, P. P., Aaronson, S. A., Potter, M., Pumphrey, J., Scott, A. & Ihle, J. N. (1985) *Cell* **41**, 685-693.

24. Barsumian, E. L., McGivney, A., Basciano, L. & Siraganian, R. (1985) *Cell. Immunol.* **90**, 131-141.

25. Dunn, T. B. & Potter, M. (1957) *J. Natl. Cancer Inst.* **18**, 587-595.

26. Howard, M., Farrar, J., Hilfiker, M., Johnson, B., Takatsu, K., Hamaoka, T. & Paul, W. E. (1982) *J. Exp. Med.* **155**, 914-923.

27. Fox, B. (1993) *Int. Immunol.* **5**, 323-330.

28. Gutman, G. A., Warner, N. L. & Lanier, L. L. (1981) *Clin. Immunol. Immunopathol.* **18**, 230-244.

29. O'Garra, A., Stapleton, G. Dhar, V., Pearce, M. Schumacher, J., Rugo, H., Barbis, D., Stall, A., Cupp, J., Moore, K., Vieira, P., Mosmann, T., Whitmore, A., Arnold, L., Haughton, G. & Howard, M. (1990) *Int. Immunol.* **2**, 821-832.

30. Ralph, P., Moore, M. A. S. & Nilsson, K. (1976) *J. Exp. Med.* **143**, 1528-1533.

31. Leo, O., Foo, M., Sachs, D. H., Samelson, L. E. & Bluestone, J. A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1374-1378.

32. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

33. Fiering, S., Northrop, J. P., Nolan, G. P., Mattila, P. S., Crabtree, G. R. & Herzenberg, L. A. (1990) *Genes Dev.* **4**, 1823-1834.

34. Schuetze, S., Paul, R., Gliniak, B. C. & Kabat, D. (1992) *Mol. and Cell. Biol.* **12**, 2967-2975.

35. Pahl, H. L., Scheibe, R. J., Zhang, D., Chen, H., Galson, D. L., Maki, R. A. & Tenen, D. G. (1993) *J. Biol. Chem.* **268**, 5014-5020.

36. Dynan, W. S. & Tijan, R. (1983) *Cell* **35**, 79-87.

37. Evans, T., Reitman, M. & Felsenfeld, G. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5976-5980.

38. Wang, C., Petryniak, B., Hu, I., Thompson, C. B., & Leiden, J. M. (1992) *J. Exp. Med.* **175**, 1391-1399.

39. Leiden, J. M. (1993) *Ann. Rev. Immunol.* **11**, 539-570.

40. Orkin, S. H. (1992) *Blood* **80**, 575-581.

41. Klemsz, M. J., McKercher, S. R., Celada, A., Van Beveren, C. & Maki, R. A. (1990) *Genes & Dev.* **4**, 1451-1453.

42. Pettersson, M. & Schaffner, W. (1987) *Genes and Dev.* **1**, 962-972.

43. Klemz, M. J., McKercher, S. R., Celada, A., Van Beveren, C. & Maki, R. A. (1990) *Cell* **61**, 113-124.

44. Ray, D. Bosselut, R., Ghysdael, J., Mattei, M., Tavitian, A & Gachelin, F. (1992) *Mol. Cell. Bio.* **12**, 4297-4304.

45. Galson, D. L., Hensold, J. O., Bishop, T. R., Schalling, M., D'Andrea, A. D., Jones, C., Auron, P. E. & Housman, D. E. (1993) *Mol. Cell. Bio.*, **13**, 2929-2941.

46. Rivera, R. R., Stuiver, M. H., Steenbergen, R. & Murre, C. (1993) *Mol. and Cell. Bio.* **13**, 7163-7169.

47. Ben-Sasson, S., Le Gros, G., Conrad, D. H., Finkelman, F. D. & Paul, W. E. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 1421-1425.

48. Zon, L. I., Gurish, M. F., Stevens, R. L., Mather, C., Reynolds, D. S., Austen, K. F. & Orkin, S. H. (1991) *J. Biol. Chem.* **266**, 22948-22953.

49. Ho, I., Vorhees, P., Marin, N., Oakley, B.K., Tsai, S., Orkin, S. H. &Leiden, J.M. (1991) *EMBO J.*, **10**, 1187-1192.

50. Merika, M. & Orkin, S. H. (1993) *Mol. Cell. Biol.* **13**, 3999-4010.

51. Shin, M. K. & Koshland, M. E. (1993) *Genes Dev.* **7**, 2006-2015.

52. Wasylyk, C., Gutman, A., Nicholson, R. & Wasylyk, B. (1991) *EMBO* **10,** 1127-1134.

53. Hentsch, B., Mouzaki, A., Pfeuffer, I., Rungger, D. &Serfling, E. (1992) *Nuc. Acid Res.* **20**, 2657-2665.

54. Lemarchandel, V., Ghysdael, J., Mignotte, V., Rahuel, C. & Romeo, P. (1993) *Mol. Cell. Biol.* **13**, 668-676.

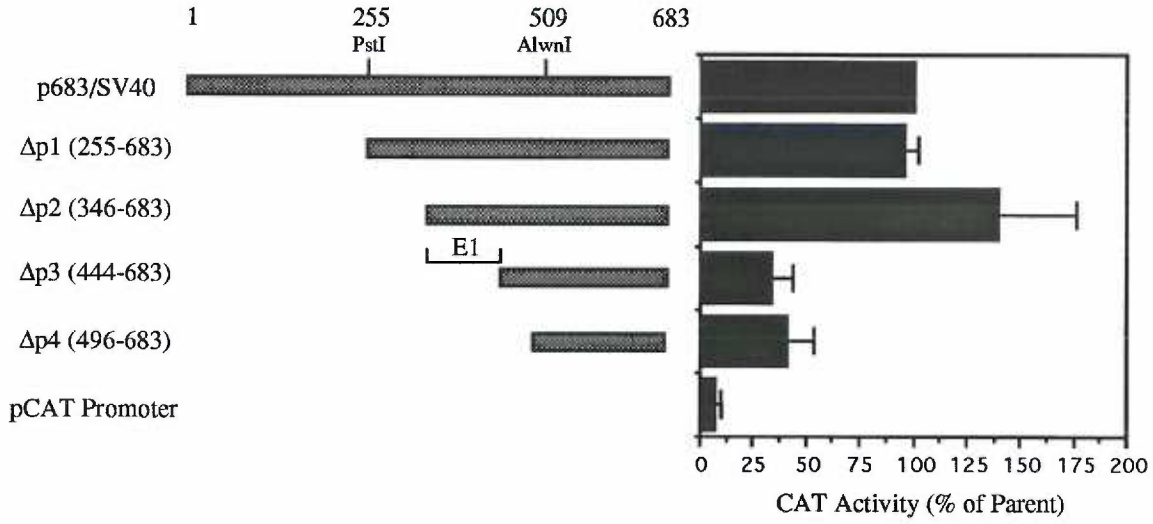
55. Gordon, J. R. & Galli, S. J. (1991) *J. Exp. Med.* **174**, 103-107.

56. Arai, N., Nomura, D., Villaret, D., Malefijt, R. D., Seiki, M., Yoshida, M., Minoshima, S., Fukuyama, R., Maekawa, M., Kudoh, J., Shimizu, N., Yokota, K., Abe, E., Yokota, T., Takebe, Y. & Arai, K. (1989) *J. Immunol.* **142**, 274-282.

Figure 1. Effect of 5' and 3' end deletions on an IL-4 intronic enhancer activity. CAT reporter gene constructs containing deletions from either the 5' (A) or 3' (B) ends of the 683 base pair Bgl II intronic fragment were generated using BAL 31 nuclease. These were compared to the parent construct (683/SV40) for the ability to enhance SV40 promoter-driven CAT gene expression in transient transfection assays. The relative enhancer activity of the truncated constructs is expressed as the mean percent of activity of the parent vector (set at 100) +/- SE of 3-5 experiments.

A

5' Deletion Constructs



B

3' Deletion Constructs

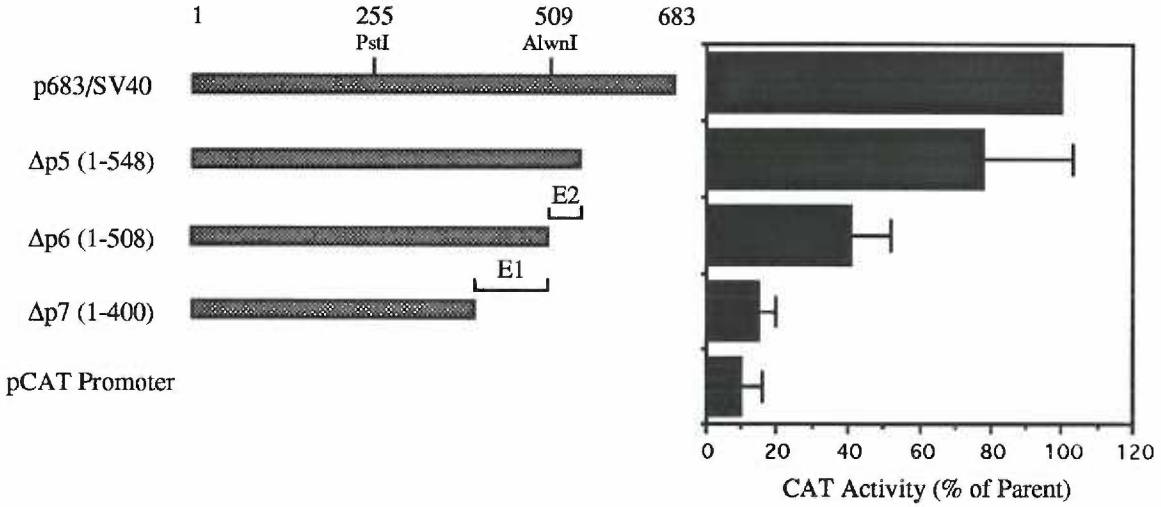


Figure 1C A construct containing intronic sequences from 353-554 demonstrates full enhancer activity when compared to activity from 683/SV40. Relative enhancer activities are expressed as a mean percent of the parent vector (set at 100) +/- SE of three experiments. The numbering of coordinates is based on designating the first base of the 5' Bgl II site as #1.

C

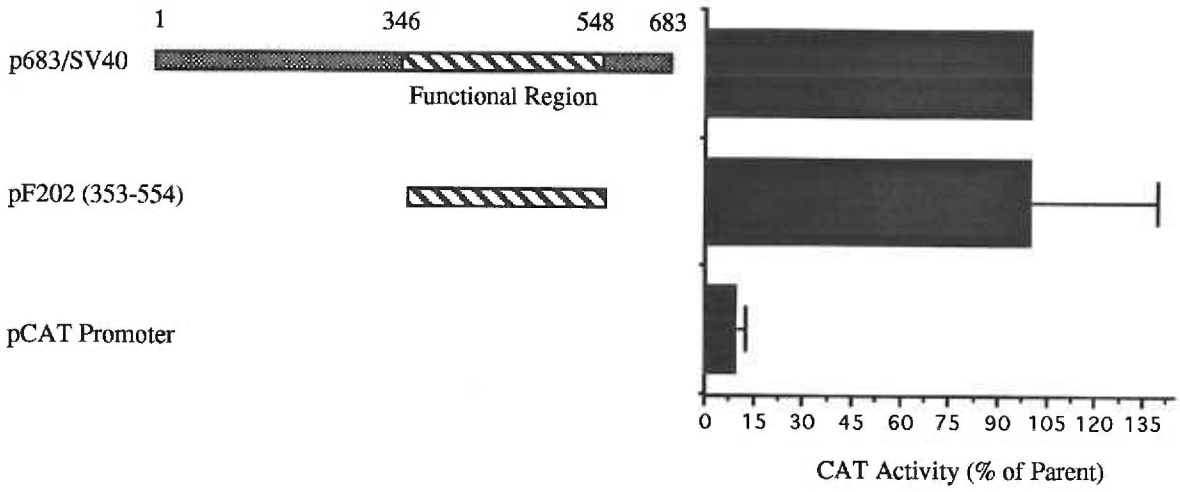

1
346
548
683
p683/SV40
Functional Region
pF202 (353-554)
pCAT Promoter
0 15 30 45 60 75 90 105 120 135
CAT Activity (% of Parent)

Figure 2. Sequence of the functionally relevant region of the IL-4 intronic enhancer (base pairs #348-548). The consensus binding sites for SP1, GATA, and ets are shown in in bold-face type. The underlined sequences are located within the E1 subregion, and the overlined sequences are within the E2 subregion. The consensus site for SP1 is, GGGGCGG (36), for GATA, WGATAR (37) and for ets, G/CAGGAA/T (38).

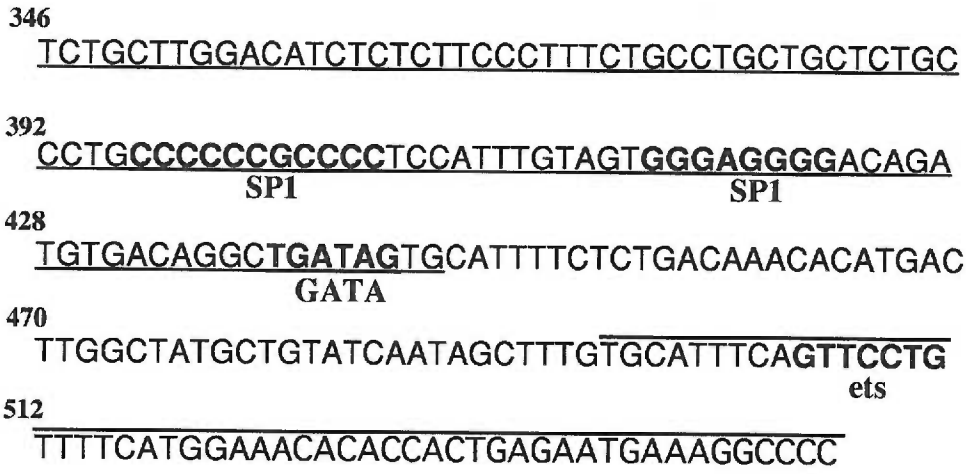

346
TCTGCTTGGACATCTCTCTTCCCTTTCTGCCTGCTGCTCTGC
392
CCTGCCCCCCCGCCCCTCCATTTGTAGTGGGAGGGGACAGA
SP1
SP1
428
TGTGACAGGCTGATAGTGCATTTTCTCTGACAAACACATGAC
GATA
470
TTGGCTATGCTGTATCAATAGCTTTGTGCATTTCAGTTCCTG
ets
512
TTTTCATGGAAACACACCACTGAGAATGAAAGGCCCC

Figure 3. Effect of mutations within the core recognition region of the IL-4 intronic SP1, GATA and ets binding sequences on enhancer activity. Single or double mutations were introduced within the SP1, GATA, and/or the ets site in Δp1. X's represent altered sites. The relative enhancer activity of the mutant constructs is expressed as a mean percent of the activity of Δp1 (set at 100) +/- SE of 3-5 experiments.

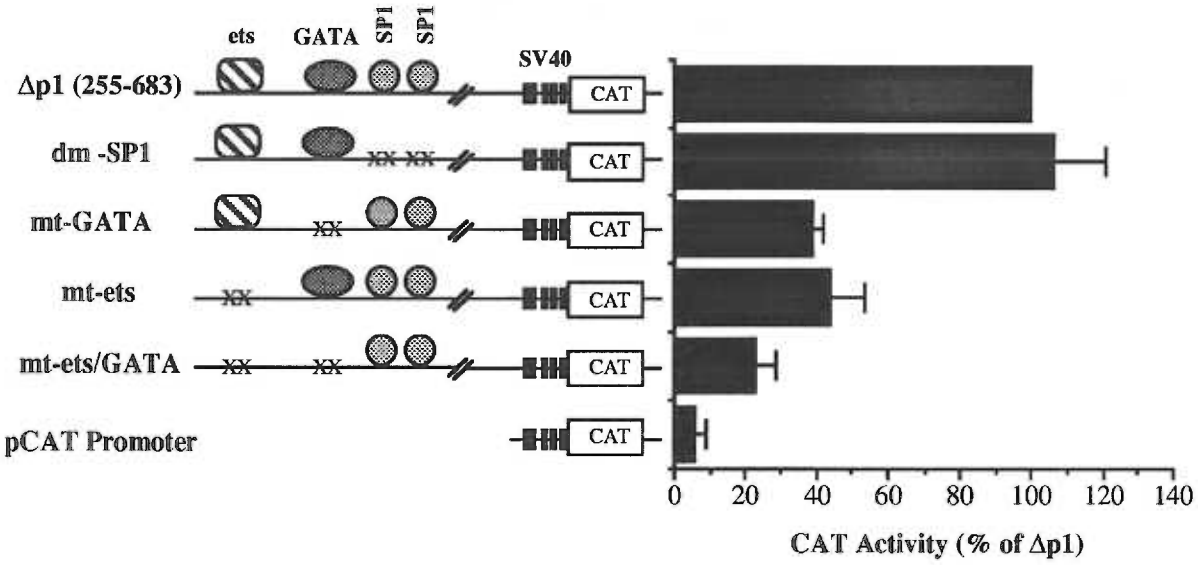

ets
GATA
SP1
SP1
SV40
Δp1 (255-683)
dm -SP1
mt-GATA
mt-ets
mt-ets/GATA
pCAT Promoter
CAT
0
20
40
60
80
100
120
140
CAT Activity (% of Δp1)

Figure 4. Both the IL-4 GATA and ets binding sequences form specific DNA/protein complexes. EMSAs were performed using 0.1 ng of ^{32}P labeled GATA (lanes 1-4) or ets (lanes 5-8) DNA probe incubated with 5 μg of ABFTL-3 nuclear extract in the absence (-) or presence of 10 ng (100-fold molar excess) of the indicated competitor DNAs. Protein-DNA complexes were resolved on a 5% tris-glycine polyacrylamide gel. m-GATA (lane 3) and m-ets (lane 7) competitor DNAs are oligonucleotides containing the same mutations that result in reduced CAT activity in functional assays as described in figure 3. The two complexes formed with the GATA probe are indicated by A and B. The single ets complex is indicated by an arrow.

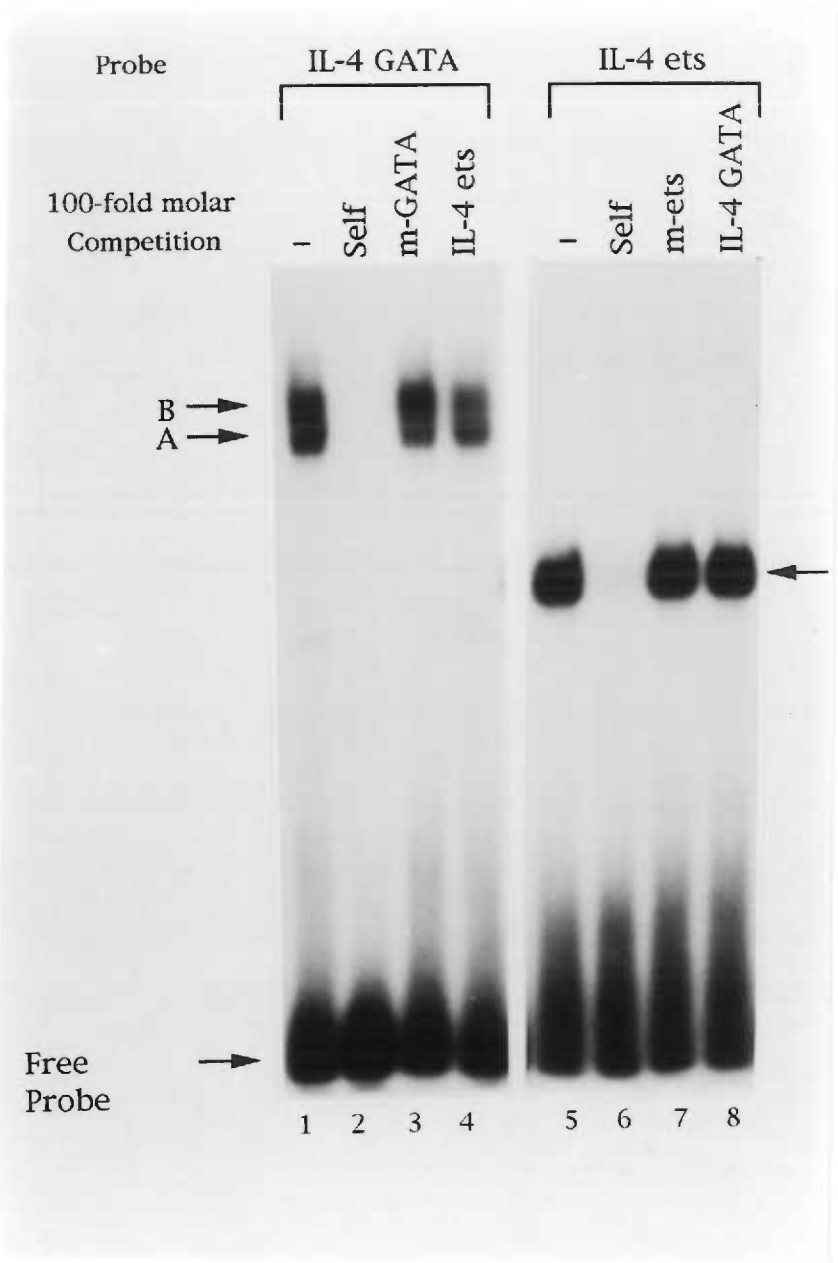

Probe
IL-4 GATA
IL-4 ets
100-fold molar Competition
–
Self
m-GATA
IL-4 ets
–
Self
m-ets
IL-4 GATA
B
A
Free Probe
1 2 3 4 5 6 7 8

Figure 5. Differential binding to the IL-4 GATA and ets sequence correlates with enhancer function. EMSAs were performed as described in Figure 4 using nuclear extracts from unstimulated (-) or stimulated (+) cells of mast (lanes 1-5), T (lanes 6-9) and B cell (lanes 10-11) lineage. CFTL 12 mast cells were stimulated with 0.75μM ionomycin for 30 minutes. EL-4 cells were stimulated with 10 ng/ml PMA. GA15 T cells were stimulated with 2C11 (1 μg/ml for 12 hours. (A) EMSA using the IL-4 GATA oligonucleotide probe. (B) EMSA using the IL-4 ets oligonucleotide probe.

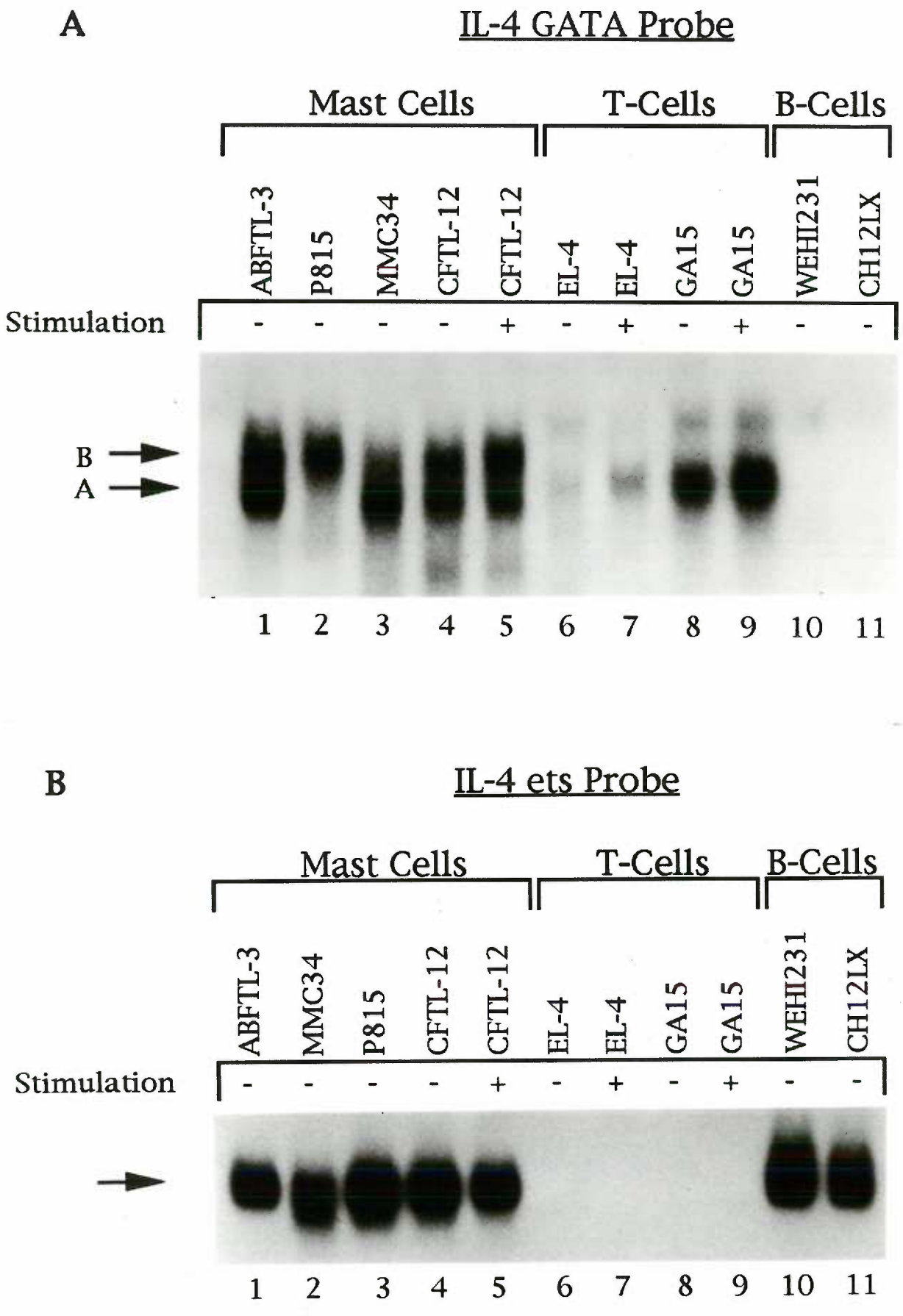

A
IL-4 GATA Probe
Mast Cells
T-Cells
B-Cells
ABFTL-3
P815
MMC34
CFTL-12
CFTL-12
EL-4
EL-4
GA15
GA15
WEHI231
CH12LX
Stimulation
- - - - + - + - + - -
B
A
1 2 3 4 5 6 7 8 9 10 11
B
IL-4 ets Probe
Mast Cells
T-Cells
B-Cells
ABFTL-3
MMC34
P815
CFTL-12
CFTL-12
EL-4
EL-4
GA15
GA15
WEHI231
CH12LX
Stimulation
- - - - + - + - + - -
1 2 3 4 5 6 7 8 9 10 11

Figure 6. GATA-1 and GATA-2 are present in ABFTL-3 cells and bind to the IL-4 intonic GATA site. One μl of anti-GATA-1 (lane 2) or anti-GATA-2 (lane 3) were preincubated with with 5 μg of ABFTL-3 nuclear extract in binding buffer for 15 minutes prior to the addition of ^{32}P labeled IL-4 GATA probe. One μl of either preimmune rabbit serum or ets specific antiserum (anti-PU.1) was used as negative controls (lanes 4 and 5, respectively).

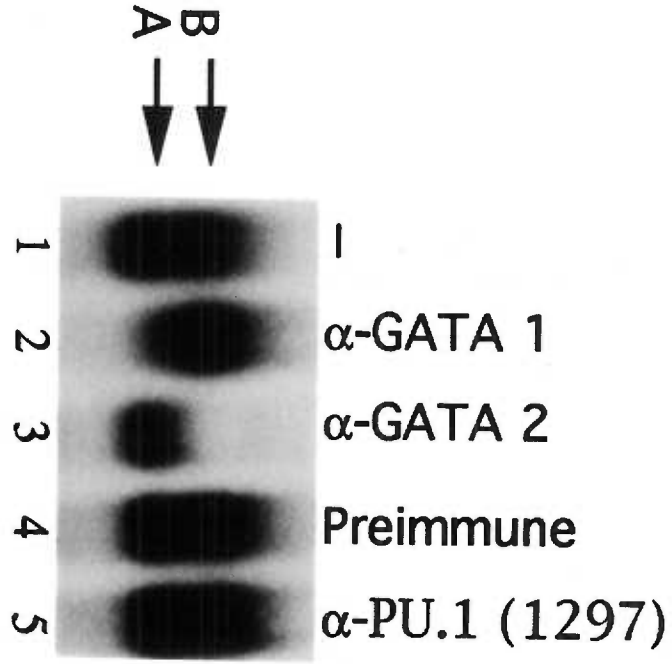
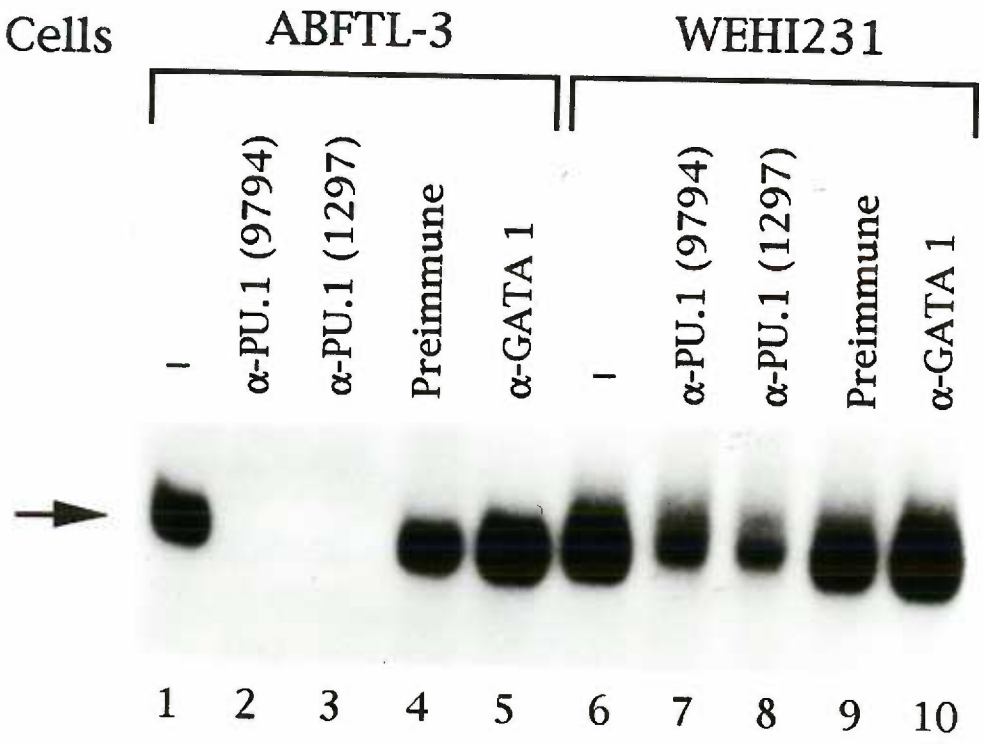

A
B
1
2
3
4
5
I
α-GATA 1
α-GATA 2
Preimmune
α-PU.1 (1297)

Figure 7. The ets family member, PU.1, is present in mast cell nuclear extracts and binds to the IL-4 ets site. 1 µl of either rabbit anti-PU.1 sera 9794 (lanes 2 and 7) or 1297 (lanes 3 and 8), was preincubated with 5 µg of either ABFTL-3 or WEHI 231 nuclear extract in binding buffer for 15 minutes prior to the additon of labeled IL-4 ets probe. 1 µl of preimmune rabbit serum (lanes 4 and 9) or anti-GATA 1 (lanes 5 and 10) were used as negative controls. (A) EMSA with ABFTL-3 mast cell nuclear extract. (B) EMSA with WEHI 231 B-cell nuclear extract.


Cells
ABFTL-3
WEHI231
-
α-PU.1 (9794)
α-PU.1 (1297)
Preimmune
α-GATA 1
-
α-PU.1 (9794)
α-PU.1 (1297)
Preimmune
α-GATA 1
1 2 3 4 5 6 7 8 9 10

Conclusion and Discussion

T and mast cells originate from a common pluripotent stem cell in the bone marrow, but commit to distinct differentiation pathways resulting in different morphological and functional characteristics. Although they are distinct, both cell types do express common cytokines that, until recently, were thought to be associated primarily with T-cell production. It is likely that the functional consequence of cytokine production by mast cells may have both unique and common effects in relation to T-cell cytokine production. One distinction may be in their range of function in that due to their fixed tissue locations mast cells and the cytokines they produce contribute to local immunological responses whereas cytokines produced by circulating T-cells may have more of a systemic effect. Because of their different locations, modes of activation, and other phenotypic characteristics, it is likely that T and mast cells would evolve some cell-type specific mechanisms in regulating the production of cytokines they share in common to best serve their role in the host. The identification of a mast cell specific enhancer element located in the second intron of the IL-4 gene supports this hypothesis (Henkel et al., 1992).

The enhancer element was initially identified in transformed mast cell-lines that constitutively express IL-4 using a DNAse I hypersensitivity mapping technique (Henkel et al., 1992). The hypersensitive site (hs) was not present in either activated EL-4 T-cells or non-transformed mast cells. Further functional characterization identified this hs region as a prototypic enhancer element that was active with both the endogenous IL-4 promoter and a heterologous SV40 promoter in several transformed mast cell-lines

that constitutively expressed IL-4. Although there was no hs in the activated non-transformed mast cell, the enhancer element did function in these cells in both uninduced and ionomycin stimulated conditions. The lack of a hs site in the non-transformed mast cells may be a result of timing in that at the time of DNAse hs analysis alterations in chromatin structure had not yet taken place. Alternatively, though not mutually exclusive, it is possible that the intronic enhancer may be a site for deregulation of IL-4 gene expression in transformed mast cells. Therefore, the intronic enhancer may not be as dominant in the non-transformed mast cells as in the transformed cells. In correlation with the DNAse I hs study, the enhancer was not functional in uninduced or PMA stimulated EL-4 T-cells. Also, this enhancer was not functional in non-IL-4 producing B-cells.

Many cellular enhancers that have been identified are characterized as multimodule complexes. Further molecular dissection of the IL-4 enhancer element identified two key modules that were important for activity. The E1 module contained two SP-1 consensus sites and a GATA binding site. The E2 module contained an ets consensus site. Mutational/functional analysis of these binding sites either alone or in combination, demonstrated that the SP-1 sites were not critical for enhancer activity, but mutation of either the GATA or ets site resulted in about a 50% reduction in enhancer activity in the ABFTL-3 transformed mast cell-line. Mobility shift studies using ABFTL-3 nuclear extracts demonstrated that specific proteins could bind to oligonucleotides containing either the GATA or ets site, whereas oligonucleotides that contained the mutant sites could not successfully compete for protein binding to the labeled wild-type DNA probe. Therefore, the loss of protein binding to the mutant sequences correlated with the loss of

function. Finally, specific antibodies to GATA-1 and GATA-2 in combination with mobility shift assays demonstrated that the these factors were present in the two protein/DNA complexes seen with various mast cell lines used in this study. In addition, specific antibodies to the ets family member PU.1 demonstrated that this factor formed the IL-4 ets complex with mast cell nuclear extracts in DNA binding studies. GATA-1 and GATA-2 were not found in T-cell GATA complexes, correlating with other studies demonstrating that GATA-1 and 2 are expressed in similar cell-types including mast cells, but not T-cells or B-cells (Orkin, 1992). The PU.1 factor was present in both mast cells and B-cells, but not T-cells, again correlating with previously published data (Dominique et al., 1992; Galson et al., 1992; Klemz et al., 1990). Therefore, the cell-restricted activity of this intronic enhancer element can be explained in part by the restricted expression of the GATA and PU.1 transcription factors in mast cells versus T-cells.

Further work with expression vectors that carry the cDNA for either GATA-1, GATA-2 or PU.1 is needed to verify *in vivo* that these factors are regulating the intronic enhancer in mast cells. One approach is to co-transfect the IL-4 enhancer reporter vector with either of the expression vectors or in various combinations into EL-4 T-cells to determine if the presence of these transcription factors is sufficient for enhancer activity. Because there may be negative elements or missing mast cell-specific factors in the T-cells, co-transfection of the various plasmids in the mast cells should also be included. In addition, expression vectors can be made that express only the DNA binding domain of the transcription factors to be used in the mast cell co-transfection studies as dominant negative mutants.

Another GATA family member, GATA-3, is not expressed in mast cells but is expressed in T-cells (Orkin, 1992). EL-4 T-cells have been shown to express GATA-3 (Ho et al., 1991). Therefore, it is unclear why the IL-4 enhancer did not give partial activity in these cells. GATA factors can bind with high affinity to the consensus WGATAR site *in vitro* (Orkin, 1992). Also, the DNA binding domain among the GATA transcription factors is very homologous (Orkin, 1992). It is possible there are*in vivo* binding differences among the GATA family members influenced by flanking DNA sequences that regulate binding specificity not detected by *in vitro* binding studies. Alternatively, there may be *trans*-activation differences among the GATA factors. Sequences outside of the highly homologous DNA binding domain are less conserved (Orkin, 1992). This may have functional significance in terms of interactions with other proteins that regulate GATA activity. Therefore, it is possible that other T-cell factors required for GATA-3 activity were unable to interact with the IL-4 enhancer. A similar explanation may also be used for why there was no partial enhancer activity in B-cells that express PU.1. One study has shown that the B-cell specific κ3' enhancer requires both PU.1 and an adjacent factor NF-EM5 for efficient *trans*-activation (Pongubala et al., 1992). A search of the IL-4 enhancer sequence did not locate a NF-EM5 binding site. Another possibility as to why there was no partial enhancer activity in T or B-cells is that there may be an NRE site(s) that blocks enhancer activity these cells.

The IL-4 ets site was unable to form any protein/DNA complexes with T-cell nuclear extracts. This was unusual since T-cells express various ets family members (Leiden, 1993). Therefore, the IL-4 ets sequence may also contribute to the cell restricted enhancer activity. It has been

demonstrated that flanking sequences surrounding the core GGA binding site can influence the binding specificity of the various ets family members (Wang et al., 1992; Wasylyk et al., 1992). Mutation of the IL-4 ets site to resemble those that bind ets factors in T-cells may alter the specificity of this intronic enhancer element. However, T-cell ets transcription factors may require other proteins for activation and therefore, the mutation of the IL-4 ets site alone may not be sufficient. Interestingly, the T-cell specific TCR-α enhancer has been shown to be regulated by several transcription factors including, GATA-3 and Ets-1 (Leiden, 1993). Therefore, mutation of the PU.1 binding site to an Ets-1 binding site in combination with the wild-type IL-4 GATA sequence may be sufficient to alter the cell restricted activity of the IL-4 intronic enhancer.

Mutation of both the GATA and ets sites did not result in a complete loss of enhancer activity. This suggested that there may be additional site(s) necessary for full enhancer activity. DNAse I footprint analysis of the functional enhancer region with ABFTL-3 nuclear extract did identify an additional footprint between the GATA and PU.1 binding sites (unpublished results). However, the footprint could be competed with an unrelated oligonucleotide. Also, a DNA probe from the footprinted region could not form a specific complex in mobility shift assays (unpublished results). Alternatively, there may be sequences within the range of DNAse I footprints of either the GATA and/or PU.1 sites that bind additional factors. An elongated IL-4 PU.1 oligonucleotide that contained additional 3' flanking nucleotides from the intronic enhancer formed a second specific complex that ran with a lower mobility relative to the PU.1 complex in mobility shift analysis (unpublished results). Competition studies demonstrated that this complex did not contain PU.1. Further work is

needed to determine if the protein(s) associated with the lower mobility complex plays a role in enhancer activity. A final explanation may be that the SP-1 sites are contributing to the partial activity in an artificial manner. It is possible that in the presence of both GATA and PU.1, SP-1 cannot bind to its consensus sites in the intronic enhancer. DNAse I footprint analysis of the functional region demonstrated a very weak footprint over the 5' SP-1 site and none over the 3' site (unpublished results). Inhibition of GATA and PU.1 binding may enable SP-1 to bind to its cognate sites in the IL-4 enhancer. SP-1 can function as a transactivator and we have demonstrated by mobility shift assays in combination with SP-1 specific antibodies that this factor is able to bind to the IL-4 SP-1 binding sequences (unpublished results). Also, a truncated construct from the functional region that retains the 5' SP-1 site only, demonstrated partial enhancer function in transient transfections in ABFTL-3 (unpublished results). Experiments to mutate both SP-1 sites in the context of the mutated GATA and PU.1 sites are needed to test this hypothesis.

It has been demonstrated that mast cells store IL-4 and other cytokines in their secretory granules (Gorden and Galli, 1991; Bradding et al., 1992; Bradding et al., 1993). This is clearly a distinct phenotypic/functional characteristic between mast and T-cells. The presence of preformed IL-4 in mast cell granules would suggest that these cells would have to maintain a certain level of IL-4 mRNA for translation into protein. Northern analysis demonstrated that non-transformed mast cells express a low basal level of IL-4 mRNA (Brown et al., 1987). The IL-4 intronic enhancer was shown to increase IL-4 promoter activity in both uninduced and ionomycin stimulated non-transformed mast cells (Henkel et al., 1992). This correlates with the *in vitro* binding data demonstrating that the GATA and

PU.1 protein/DNA complexes formed under both conditions. Therefore, it is likely that the intronic enhancer plays a role in maintaining a basal level of IL-4 expression for storage in the cytoplasmic granules. It does not appear that there are any qualitative or quantitative differences with either the GATA or PU.1 protein/DNA complexes in uninduced and induced extracts. However, there may be some postranslational modifications that were not detected by mobility shift assays. An ionomycin inducible element in the IL-4 promoter has been defined in mast cells (Weiss et al. manuscript in preparation). Future studies will be needed to understand how the intronic enhancer interacts with both the basal and stimulated IL-4 promoter elements.

Comparison of the mouse IL-4 intronic enhancer to the equivalent region within the human IL-4 gene demonstrates that both the GATA and PU.1 sites, including several flanking sequences are 100% conserved. The entire functional region is 82% homologous between mouse and human IL-4 gene. This level of conservation within an intron between two distinct species, suggests that this region may have functional significance in human mast cell regulation of IL-4. Unfortunately, it is very difficult to culture freshly isolated human mast cells and there appears to be only one human mast cell-line that was cloned from a patient with mast cell leukemia (Butterfield et al., 1988). We are currently determining whether or not these cells make IL-4. They have been shown to express TNF-α and IL-1β constitutively (Sillaber et al., 1993).

Most of this study was done with ABFTL-3 transformed mast cells primarily because they were easy to maintain in culture and were highly transfectable. There is evidence to suggest that IL-4 may act as an autocrine growth factor for these cells (M. Brown unpublished data).

From this standpoint these cells were of interest because they may serve as good model for understanding tumor development through deregulated expression of a growth factor. On the other hand, it has been noted from tissue samples containing mast cells that atopic individuals demonstrated higher levels of IL-4 production both intracellularly and extracellularly than normal patients (Bradding et al., 1992; Bradding et al., 1993; Hunt et al., 1992). As mentioned above, it is possible that this enhancer element is a target for deregulated expression of IL-4. Comparison of the DNA binding data of both transformed and non-transformed mast cell nuclear extracts does not indicate any apparent differences in the GATA and PU.1 protein/DNA complexes. Again, it is possible that there are modifications undetected by these assays and/or there are other factors associated with increased enhancer activity not yet defined. There is evidence demonstrating that there is higher IL-4 promoter activity in the unstimulated transformed mast cells relative to the unstimulated non-transformed mast cells (unpublished results). This would suggest that the promoter may be a potential target for deregulation in addition or alternatively to the IL-4 intronic enhancer. Further work is required to determine if there is any differential regulation of the IL-4 intronic enhancer in normal versus transformed mast cells.

IL-4 may play a variety of roles in local inflammatory responses mediated by mast cells. IL-4 could potentially modulate mast cell function as well as other IgE receptor positive leukocytes *eg.* induce IgE production by B-cells and up-regulate expression of the low affinity IgE receptor (CD23) on T-cells, B-cells, macrophages and eosinophils. IL-4 may act as an autocrine growth factor for mast cells that leads to increased mast cell numbers during an inflammatory response. Mast cell IL-4 may be

important in regulating leukocyte infiltration by augmenting adhesion molecules e.g. V-CAM-1, on endothelial cells. Also, IL-4 secreted by mast cells can have direct effects on the infiltrating leukocytes, including the determination of the T-helper phenotype.

The ability of IL-4 and other cytokines to regulate endothelial cells, fibroblasts and infiltrating leukocytes, would place the mast cells at the center of many local inflammatory reactions initiated by injury, infections, allergens or chronic diseases. Understanding the role of IL-4 in mast cell mediated responses as well as determining the molecular mechanisms that regulate production of this cytokine in these cells, may be important in developing specific anti-cytokine therapies to inhibit mast cell activity in both allergic and pathological/chronic inflammatory conditions. Because regulation of IL-4 in mast cells utilizes an enhancer element not associated with T-cell IL-4 gene expression, it may be possible to produce inhibitory agents that will target the expression of this cytokine specifically in mast cells.

References

Bradding, P., I.H. Feather, P.H. Howarth, R. Mueller, J.A. Roberts, K. Britten, J.P.A. Bews, T.C. Hunt, Y. Okayama, C.H. Heusser, G.R. Bullock, M.K. Church and S.T. Holgate. (1992), "Interleukin 4 is localized to and released by human mast cells." J. Exp. Med., **176**: 1381-1386.

Bradding, P., I.H. Feather, S. Wilson, P.G. Bardin, C.H. Heusser, S.T. Holgate and P.H. Howarth. (1993), "Immunolocalization of cytokines in the nasal mucosa of normal and perennial rhinitic subjects." J Immunol, **151**: 3853-3865.

Brown, M.A., J.H. Pierce, C.J. Watson, J. Falco, J.N. Ihle and W.E. Paul. (1987), "B cell stimulatory factor-1/interleukin-4 mRNA is expressed by normal and transformed mast cells." Cell, **50**: 809-818.

Butterfield, J. H., D. Weiler, G. Dewald and G. J. Gleich. (1988), "Establishment of an immature mast cell line from a patient with mast cell leukemia." Leukemia Res., **12**: 345-349.

Dominique, R., R. Bosselut, J. Ghysdael, M. Mattei, A. Tavitian and F. Moreau-Gachelin. (1992), "Characterization of spi-B, a transcription factor related to the putative oncoprotein spi-1/PU.1." Mol. Cell. Bio., **12**: 4297-4304.

Galson, D. L., J. O. Hensold, T. R. Bishop, M. Schalling, A. D. D'Andrea, C. Jones, P. E. Auron and D. E. Housman. (1992), "Mouse β-globin DNA-binding protein B1 is identical to a proto-oncogene, the trancription factor spi-1/PU.1, and is restricted in expression to hematopoietic cells and the testis." Mol. Cell. Bio., **13**: 2929-2941.

Gordon, J. R. and S. J. Galli. (1991), "Release of both preformed and newly synthesized tumor necrosis factor a (TNF-a)/cachectin by mouse mast cells stimulated via the FcεRI. A mechanism for the sustained action of mast cell-derived TNF-α during IgE-dependent biological rsponses." J. Exp. Med., **174**: 103-107.

Henkel, G., D. L. Weiss, R. McCoy, T. Deloughery, D. Tara and M. A. Brown. (1992), "A DNase I-hypersensitive site in the second intron of the murine IL-4 gene defines a mast cell-specific enhancer." Journal of Immunology, **149**: 3239-46.

Ho, I., P. Vorhees, N. Marin, B.K. Oakley, S. Tsai, S.H. Orkin and J.M. Leiden. (1991), "Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor α gene." EMBO, **10**: 1187-1192.

Hunt, T.C., C. Heusser, R. Mueller, G. Bullock, Y. Okayama, L. K. Ashman and M. K. Church. (1992), "Human lung mast cells produce interleukin-4." J. Allergy Clin. Immunol., **89**: 245 (abstr.).

Klemz, M.J., S.R. McKercher, A. Celada, C. Van Beveren and R.A. Maki. (1990), "The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene." Cell, **61**: 113-124.

Leiden, J.M. (1993), "Transcriptional regulation of T-cell receptor genes." Ann. Rev. Immunol., **11**: 539-570.

Orkin, S.H. (1992), "GATA-binding transcription factors in hematopoietic cells." Blood, **80**: 575-581.

Pongubala, J.M.R., S. Magulapalli, M.J. Klemsz, S.R. McKercher, R.A. Maki and M.L. Atchison. (1992), "PU.1 recruits a second nuclear factor to a site important for immunoglobulin κ 3' enhancer activity." Mol. Cell. Bio., **12**: 368-378.

Sillaber, C., D. Bevec, J. H. Butterfield, C. Heppner, R. Valenta, O. Scheiner, D. Kraft, K. Lechner, P. Bettelheim and P. Valent. (1993), "Tumor necrosis factor α and interleukin-1β mRNA expression in HMC-1 cells: differential regulation of gene product expression by recombinant interleukin-4." Experm. Hemat., **21**: 1271-1275.

Wang, C., B. Petryniak, I. Hu, C.B. Thompson and J.M. Leiden. (1992), "Evolutionary conserved ets family members display distinct DNA binding specificities." J. Exp. Med., **175**: 1391-1399.

Wasylyk, C., J. Kerckaert and B. Wasylyk. (1992), "A novel modulator domain of Ets transcription factors." Genes Dev., **6**: 965-974.