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Regulation of IL-4 production in mast cells: a paradigm for cell-type-specific gene expression

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Summary: The role of interleukin (IL)-4 as an important immunomodulatory cytokine is well established. IL-4 exhibits a highly restricted pattern of expression by cells of distinct lineages. The cell types that produce IL-4 are located in anatomically distinct locations (e.g. circulating T cells vs. fixed tissue mast cells) and thus have access to different IL-4-responsive target cells. In addition, these cells appear to regulate IL-4 expression in cell-type-specific ways. These findings suggest that an understanding of IL-4 gene regulation in T and mast cells could provide the means to specifically control IL-4 release in a lineage- and site-specific manner. In this article we review the current knowledge regarding the cell-type specific regulation of IL-4 gene expression in mast cells and compare this to what has been defined in T cells. We show that there are distinct yet parallel events that control developmentally determined chromatin modifications, allowing accessibility of the locus, and provide the potential for transcription. In differentiated cells, a subset of unique cell activation signals initiates the cascade of events that lead to transcriptional activation of the IL-4 gene.

Introduction

Interleukin (IL)-4 plays an important role in protective immunity as well as in mediating pathologic immune responses

Interleukin 4 is one of a number of cytokines produced by activated mast cells. Though originally described as a T-cell-derived factor that influenced B-cell growth, it is now known that IL-4 plays a predominant role in many immune and inflammatory reactions (for review see (1)). Two activities of this cytokine have been studied in great detail and are well described: IL-4 induces selective IgG1 and IgE isotype switching in B cells and promotes the differentiation of naïve $CD4^+$ T cells into a subset of T helper cells that express IL-4 as well as IL-5, IL-10 and IL-13 (2–4). These cytokines and Ig isotypes are hallmarks of a Th2 response and play a protective role in immunity to extracellular pathogens (2, 5). Th2 responses also serve a regulatory function by controlling the potentially harmful effects of excessive Th1-cell activity in

cell-mediated immunity to intracellular pathogens and in autoimmune reactions.

IL-4 production is highly restricted

IL-4 is produced by only a small subset of immune cells. In addition to $CD4^+$ Th2 cells, some $CD8^+$, $NK1^+$ and γδ T cells express IL-4 upon antigen activation (6–10). Cross-linking of the FcεRI in mast cells, basophils and eosinophils also results in significant IL-4 release (11–16). A recent report documents that neutrophils also express small but measurable amounts of IL-4 mRNA independent of activation (17).

There are notable differences in the developmental events that lead to the expression of IL-4 in Th2 cells and mast cells. The production of IL-4 by Th2 cells is more stringently regulated and is a reflection of the complexity of T-cell differentiation (for review see (18)). T-cell precursors arise from the same hematopoietic stem cells as mast cells. However, T-cell development involves trafficking and maturation in specialized regions of the thymus, followed by exit to the periphery. In the thymus T cells acquire expression of either CD4 or CD8 and are selected, at least in part, for the ability to discriminate between self and non-self antigens. It is not until the T cells leave the thymus, travel to the periphery and encounter antigen in secondary lymphoid organs that they complete their differentiation. If antigen priming occurs in the presence of IL-4, $CD4^+$ T cells that express the appropriate T-cell receptor develop the potential to produce relatively large amounts of IL-4. Most IL-4 transcription initiates only after antigen priming and subsequent antigen restimulation (19–21).

In contrast, mast cells do not require differentiation in such a specialized tissue environment in order to express IL-4. Considered members of the myeloid lineage, these cells undergo significant differentiation within the bone marrow microenvironment under the influence of cytokines such as stem cell factor (SCF) and IL-3 (22–24). It is thought that mast cells complete their differentiation within their resident tissue site (25). IL-4-producing mast cells can be differentiated *in vitro* from pluripotent bone marrow stem cells in the presence of SCF and IL-3. This suggests that acquiring the potential to express IL-4 can occur early in differentiation and does not require the terminal differentiation steps that take place in the tissues. Unlike IL-4 expression in T cells, mast cell IL-4 production is not dependent on prior exposure to antigen. Initial cross-linkage of the high affinity IgE receptor results in release of preformed IL-4 stored in granules within seconds of cell activation and initiation of new transcription within 15 min (26–28). Thus, the regulation of IL-4 expression by mast cells appears to be much less stringent than by T cells.

Mast cell-derived IL-4 may influence both innate and adaptive immune responses

Mast cells have been studied as the effector cells in immediate type hypersensitivity reactions and are intimately involved in respiratory and dermal allergic responses as well as asthma (29). However, their widespread distribution and their ability to produce inflammatory mediators, including cytokines, suggests they play a broader role in both protective and pathologic immune responses. Studies with mast cell-deficient mice have confirmed that mast cells play a protective role in bacterial infections (30, 31) and may also be involved in the inflammatory processes that underlie neuroinflammatory diseases such as multiple sclerosis (32).

Mast cells have been identified in the gastrointestinal and respiratory tracts and the skin (33). At these sites they are often located perivascularly where release of mast cell-derived mediators and cytokines can influence cell trafficking (33–35). In addition, they are present in sites considered immunologically privileged such as the eye and central nervous system where they are closely associated with nerves (34). IL-4 produced by mast cells at these local sites may be important for maintaining tolerance by downregulating Th1 responses.

Though generally considered fixed tissue cells, two recent studies have demonstrated that mast cells can migrate to secondary lymphoid organs (36, 37). This information, taken together with the observation that mast cells express IL-4 without antigen-specific activation, indicates mast cells are perfectly positioned to provide the earliest source of IL-4 to naïve T and B cells during initial antigen encounter. Antigen priming of naïve $CD4^+$ T cells in the presence of IL-4 will initiate and amplify differentiation to a Th2 phenotype. Mast cell-derived IL-4 may also enhance B cell proliferation in the germinal centers of secondary lymphoid organs. In addition, in concert with signals delivered through CD40L expressed on mast cells, it induces isotype switching to IgG and IgE (38).

IL-4 gene regulation

The ability of mast cells to express IL-4 (and other inducible mast cell-specific genes) is dependent on at least two temporally distinct events: 1) developmentally regulated chromatin remodeling of the *IL-4* gene locus; and 2) cell activation leading to release of preformed IL-4 and an increase in the rate

of IL-4 transcription. Cross-linking of the high affinity Fcε receptor (FcεRI) is the best-studied mode of mast cell activation. IgE-antigen complexes binding to the FcεRI results in cell activation, release of histamine and the expression of a number of inducible genes including IL-4. Receptor-mediated signal transduction leading to IL-4 expression can be bypassed using the calcium ionophore, ionomycin (13). Fc receptor-independent mechanisms of mast cell activation also exist and include signaling by complement components, neuropeptides and bacterial products such as FimH (39–41). However, the ability of these to induce *IL-4* gene expression has not been established. We have shown that IL-4 is regulated at the level of transcription by measuring transcription initiation rates using nuclear runon assays (28). New transcription is detected within 10 min of stimulation and reaches peak levels by 30 min. The following discussion will first focus on a description of *cis*- and *trans*-acting elements that regulate IL-4 transcription. We will then discuss recent findings that provide insight into how mast cells achieve accessibility of the *IL-4* gene locus during development. These events are critical for an IL-4-producing phenotype and provide the potential for *IL-4* gene expression in cells that receive the appropriate activation signals.

Transcriptional regulation: promoter proximal elements that regulate gene transcription

We have utilized a murine mast cell line, CFTL15, as well as short-term bone marrow mast cell lines (BMMC) to study the transcriptional regulation of *IL-4* gene expression. In addition to IL-4, both types of cells express FcεRI and c-kit (the SCF receptor) and exhibit characteristic granule staining with metachromatic dyes such as Giemsa or Toluidine blue (32, 42, 43). Initial studies focused on identification of sequences within 800 base pairs of the transcription initiation site (TIS) (conventionally designated +1) that regulate transcriptional activation. *CAT* reporter gene constructs containing sequences spanning the entire −800 to +5 region or containing various 5′ deletions of these sequences, were tested in transient transfection assays for the ability to exhibit *CAT* gene activity (44). The activity of these constructs in both unstimulated and stimulated (through the FcεRI or with ionomycin) CFTL15 cells was evaluated. These experiments demonstrate that the IL-4 proximal promoter mediates inducible transcription and that both positive and negative elements influence transcription. The most important region lies within −87 base pairs of the TIS. Mutational analysis of the region indicates that sequences between −87 and −70 mediate inducible expression. The critical sequences form an NF-AT binding site, termed P1, previously defined by us and others as important for inducible transcription in T cells. P1 is one of five NF-AT binding sites located within 300 base pairs of the TIS, designated P0-P4 (See *Fig. 1*). However, as described below, several features of the factor that associates with P1 in mast cells, including its regulation, size and distinct interaction partners, suggest the possibility that it is not the same as T-cell NF-AT.

Nuclear factor of activated T cells is a mast cell transcription factor

NF-AT (nuclear factor of activated T cells) was first described as a protein that regulated the activation-induced transcription of the *IL-2* gene in T cells (45). The term is now used to denote a family of transcription factors that regulate the activation-dependent expression of many genes that function both within and outside the immune system (for review see (46)). These proteins are not restricted to T cells as originally thought, but are widely distributed in many cell types including mast cells. At least five distinct genes encode the various family members [designated *NF-AT 1*(*p*), *NF-AT2*(*c*), *NF-AT3* (*c3, x*), *NF-AT4* and *NF-AT5*] (46, 47). Several isoforms of these have been described as well. Family members exhibit homology within their DNA binding domains and in conserved serine/proline-rich motifs. All are distantly related to the rel family of transcription factors which include NF-κB p50 and p65. Most NF-AT family members exist as latent cytoplasmic factors that undergo calcineurin-dependent dephosphorylation upon cell stimulation, a process inhibited by the immunosuppressive drugs cyclosporin and FK506. Dephosphorylation unmasks a nuclear localization signal that allows shuttling to the nucleus and subsequent association with AP-1 and relevant gene elements. Nuclear residence is also regulated by kinases whose action results in nuclear export (48). In fact, the duration of NF-AT residence in the nucleus may play a role in its ability to activate specific genes.

Whether each NF-AT factor acts to regulate specific genes in various cell types or each is partially redundant is unknown. The ability to ascribe gene-specific functions is hindered by the similar *in vitro* binding and transactivation activities of these proteins with promoter elements from a variety of NF-AT regulated genes. Yet, several lines of evidence support the idea that NF-AT family members have gene-specific and/or cell-type specific activities. For example, NF-AT family members have very diverse sequences outside the homology regions providing the opportunity for interaction with unique cofactors that direct gene-specific activity (46). The most compelling argument for gene-specific activities for NF-AT comes from gene expression studies in mice bearing tar-

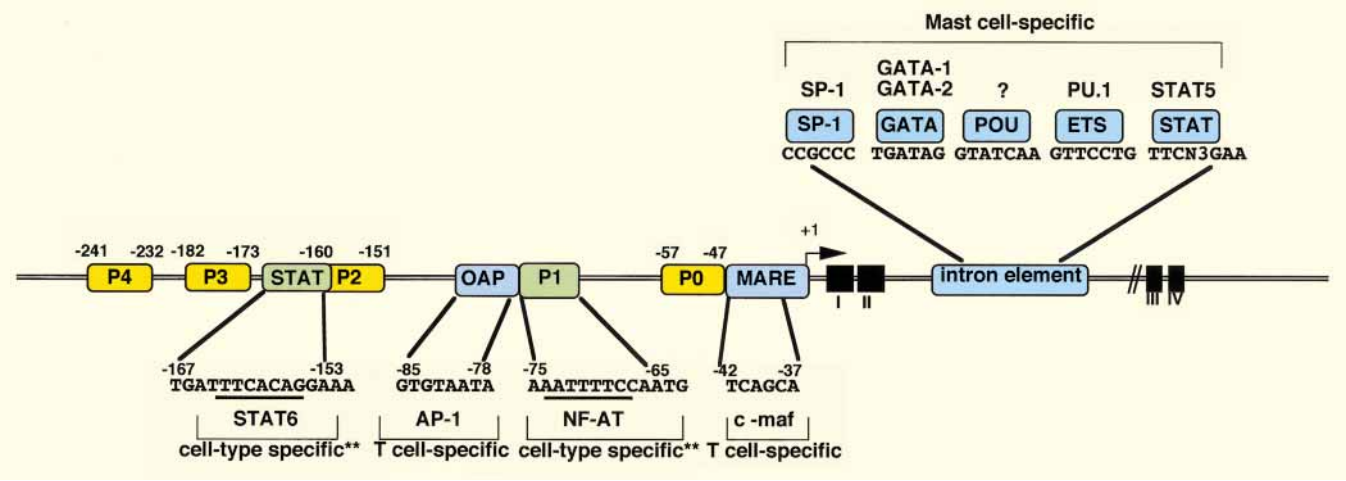

***Fig. 1.* Cell type specific *cis* and *trans*-acting elements regulate the expression of the murine *IL-4* gene.** Exons are denoted by roman numerals. DNA elements that have been defined by mutagenesis or EMSA binding assays to play a role in IL-4 gene expression are shown as colored boxes. Co-ordinates are given relative to +1, the transcription initiation site. P1–P5 designate NF-AT binding sites within the proximal IL-4 promoter. The OAP and MARE site function as T-cell-specific regulatory elements. The intron element appears to act as a mast cell specific-regulatory element. **P1 is the target of distinct NF-AT proteins in T and mast cells. The STAT binding site associates with distinct STAT6 isoforms in T and mast cells.

geted deletions in the *NF-AT1*, *NF-AT2* and *NF-AT4* genes. For example, the expression of IL-2 is only slightly affected in *NF-AT2*$^{-/-}$ mice. However, IL-4 production by activated T lymphocytes is severely impaired in these mice, indicating that the *IL-4* gene is one target of NF-AT2 (49). T cells from *NF-AT1*$^{-/-}$ mice exhibit delayed kinetics of IL-4 expression but overall IL-4 expression is enhanced (50, 51). In contrast, IL-4 production by NF-AT4-deficient T cells is normal (52).

DNA–protein interaction assays (electrophoretic mobility shift assays) and supershift analyses with NF-AT1 and NF-AT-2-specific antibodies confirmed that an NF-AT-like factor can associate with the −87 to −70 *IL-4* promoter (P1) element (44). Our analysis of the NF-AT that associates with the P1 element in mast cells demonstrates that it is unique from the NF-AT molecule associated with the *IL-4* gene in T cells. First, mast cell NF-AT binding activity is present in the nucleus of unstimulated cells, suggesting this factor does not undergo activation-dependent translocation. Secondly, rather than inhibiting nuclear transport, CSA inhibits DNA-NF-AT interactions. It is not associated with AP-1 as in T cells. Finally, oligonucleotide affinity chromatography followed by SDS PAGE experiments demonstrates that the NF-AT-related mast cell factor has a molecular weight of 41 kD, much smaller than the T-cell factors, which range in size from ~80 to 100 kD.

To further characterize mast cell expression of NF-AT, a mast cell cDNA library was screened with a DNA probe encoding a portion of the NF-AT1 rel homology domain. A few clones were identified corresponding to *NF-AT1*, *NF-AT3* and *NF-AT4*. The majority of clones selected encoded two isoforms of NF-AT2 (NF-ATc), designated NF-AT2.α and NF-AT2.β. These molecules differ only in their amino terminal sequence. Despite minimal discrepancies in the coding region, there are striking tissue- and cell-type-specific differences in expression patterns (53). RNAse protection assays with isoform-specific probes demonstrated that constitutive expression of NF-AT2.α occurs in the spleen, whereas β is expressed at high levels in the spleen, heart and kidney. Detection of NF-AT2.α mRNA is strictly dependent on cell activation in both T and mast cells. In contrast, NF-AT2.β is expressed at very low levels in all cells, but only responds to mast cell activation signals delivered through the FcεRI or via calcium ionophore signaling.

Studies in progress are directed towards understanding the function of these isoform-specific regions and determining the molecular basis for their distinct expression patterns. We hypothesize that the isoform-specific regions are uniquely responsive to the distinct signals transduced through the FcεRI and the TCR in their respective cell types. Previous *NF-AT2* gene targeting experiments deleted exons encoding sequences common to all NF-AT2 isoforms. This results in complete loss of expression of all isoforms. If the α and β isoforms are encoded on distinct exons as we predict, targeted de-

letions of these individual regions would provide important information on the *in vivo* function of each molecule. Because we still do not know the relationship of either of these isoforms to the factor that associates with the mast cell *IL-4* gene *in vivo*, such information will be critical for determining a role, if any, in mast cell IL-4 gene expression.

Th2-associated transcription factors c-maf, STAT6 and GATA3 are not involved in IL-4 expression by mast cells

Much effort has focused on identifying factors that are differentially expressed or activated during Th development in order to understand the molecular basis of polarized cytokine expression by helper T cells. Three transcription factors have been implicated in the development of a Th2 response: STAT6, GATA3 and c-maf (54). STAT6, a member of the signal transducer and activator of transcription family of proteins is activated via phosphorylation in response to IL-4-receptor interactions (55, 56). Its expression is not restricted to Th2 cells, but most $CD4^+$ $STAT6^{-/-}$ T cells fail to develop into Th2 cells and thus are unable to express significant amounts of IL-4 (57–59). This reflects the loss of an intact IL-4 signaling pathway during antigen priming necessary for Th2 differentiation. The precise mechanism of STAT6 action in T-cell development and IL-4 expression has not been discerned. A STAT6 consensus binding site has been identified within the proximal *IL-4* promoter (see *Fig. 1*). However, it is likely that STAT6 does not influence transcriptional initiation directly. This conclusion is based on experiments showing that mutation or deletion of the *IL-4* gene STAT6 site has no effect on reporter gene activity in differentiated Th2 cells (60, 61). Rather, it is likely it exerts its influence on IL-4 expression early in T-cell development. Bone marrow mast cells from $STAT6^{-/-}$ mice express similar levels of IL4 mRNA and protein when activated, as do the wild-type cells (62). Thus, mast cell *IL-4* gene expression is independent of STAT6 signaling pathways.

c-maf was identified in a genetic screen of NF-AT1-associated factors (63). It is a distant cousin of the AP-1 family of transcription factors and is selectively expressed in Th2 but not Th1 cells. It appears to have an important and direct role in IL-4 transcription. c-maf-deficient T cells have significantly diminished IL-4 expression but produce normal levels of other Th2 type cytokines such as IL-13 (64). Transfection of a c-maf expression construct into B cells can induce IL-4 production, albeit at low levels (63). c-maf binds to a consensus *MARE* binding site located in the proximal *IL-4* promoter, adjacent to the P0 NF-AT binding site. Mutation of this site within the context of an IL-4 reporter gene context abolishes inducible activity in transiently transfected Th2 cells. In contrast, mast cells do not express c-maf mRNA. Furthermore, the *MARE* site does not appear to play a role in inducible *IL-4* gene expression based on the observation that mutation of the *MARE* site has no effect on *CAT* gene expression in mast cells.

GATA-3 is a member of the GATA family of transcription factors. These transcription factors play critical roles in the development of several hematopoietic cell lineages. GATA-3 is selectively expressed in Th2 cells and appears to be essential for Th2 development (65). In fact, ectopic GATA-3 expression by naïve $CD4^+$ T cells can compensate for the lack of STAT6 expression and lead to Th2 development (66, 67). Like STAT6, it is not known how GATA-3 exerts its influence. There are no known functional *GATA* sites within the proximal *IL-4* promoter although distal sites have been described (68). As discussed below, STAT and GATA-3 may not directly impact inducible IL-4 transcription but rather may play an important role in chromatin remodeling of the *IL-4* locus during development. GATA3 expression is restricted to T cells, and therefore it cannot play a role in mast cell IL-4 expression. However, GATA-1 and GATA-2 are constitutively expressed in mast cells and may exert similar effects in these cells (69).

Developmental influences on *IL-4* gene expression

The regulated activation and binding of transcription factors such as NF-AT and c-maf is not sufficient to achieve gene expression of IL-4. In order for these factors to associate with specific regulatory regions, the gene must be in an accessible configuration. Chromatin, the highly ordered structure of chromosomes, arises from DNA-protein interactions that control packaging of the DNA. It is now clear that developmental events associated with establishing a particular cell lineage can alter (or remodel) the local chromatin structure of cell type-specific genes and modulate their accessibility to transcription factors (for review see (70)).

Chromatin accessibility determinants

The reversible processes of histone acetylation and DNA demethylation contribute to chromatin remodeling and an accessible gene locus (for reviews see (71, 72)). Chromatin is organized into nucleosomal subunits. Each nucleosome consists of 146 bp of DNA wrapped around two molecules each of the histone proteins H2A, H2B, H3 and H4 and a single linker protein, H1. The amino terminal tails of histones extend outside of the nucleosome, where they interact with other proteins as well as with DNA. The tail domains are lysine rich and are targets of a class of enzymes termed

histone acetyl transferases. Acetylation of H4, for example, results in a significant reduction in affinity of H4 for DNA and presumably destabilizes the normally condensed nucleosome configuration, allowing it to become more accessible. Local histone acetylation is also associated with gene transcriptional activity and is another marker of accessible chromatin. Methylation of DNA occurs on cytosine residues within CpG dinucleotides and correlates with gene silencing. Both methylated and demethylated DNA are assembled into nucleosomes. Two major mechanisms have been proposed to account for the block of transcription by methylation. The addition of a methyl group within a transcription factor binding site may interfere with protein binding. Methyl CpG dinucleotides may be targeted by repressor proteins such as MeCP1 and MeCP2. These have been shown to bind irrespective of sequence and recruit histone acetyl transferases to specific DNA regions. Alternatively, binding of such repressors may directly block transcription factor binding.

The accessibility of a specific gene segment can be assayed *in vitro* by measuring susceptibility to restriction enzyme digestion or to endonucleases such as DNAse I. DNAse I hypersensitivity site analysis exploits the fact that the native chromatin configuration of genes with the potential to be transcribed in a particular cell type has a more accessible conformation. Thus, treatment of isolated cell nuclei with limiting concentrations of DNase I will generate double-strand nicks in the DNA regions that are more accessible. Southern blot analysis of the DNAse I-induced cleavage sites within the purified DNA can be used to map the sites of relatively "open" chromatin. CpG methylation states are assessed by using restriction enzymes that distinguish between methylated and demethylated CpGs within the recognition site or through techniques such as bisulfonate sequencing which can identify all methylated CpGs.

Chromatin remodeling of the *IL-4* gene locus in Th2 cells

Both types of analysis have shown that the mechanisms underlying the acquisition of an accessible IL-4 gene locus exhibit lineage-specific features. A $CD4^+$ T helper cell must exit the thymus and encounter antigen in the secondary lymphoid organs before it begins to differentiate to the Th2 (or Th1) phenotype and initiates the process of chromatin remodeling of IL-4 (or interferon (IFN)-γ). While IL-4 and the associated STAT6 signaling pathways are not absolutely required for Th2 development and IL-4 production (IL-4 independent pathways for T cell IL-4 production do exist), it is clear that if present, the magnitude of the response is greatly enhanced (66, 73). The way STAT6 influences these events is still unclear, but it may do so through direct influence on remodeling of the *IL-4* locus. Several defined STAT6 binding sites are present at distal sites from the promoter, both 5′and 3′ of the *IL-4* gene as well as within the second intron (74, 75). STAT6 is able to recruit and associate with CBP and p300, two related co-activators that exhibit histone acetyl transferase activity (76, 77). The inability to express IL-4 due to a STAT6 deficiency in T cells correlates with the appearance of DNAse I hypersensitive sites within the *IL-4* gene locus (78). The lack of IL-4 production and an inaccessible locus can be overcome by treating $STAT6^{-/-}$ T cells with trichostatin A and 5-azacytidine, inhibitors of histone deacetylase and DNA methylation, respectively (73). STAT6 may also act indirectly by inducing the expression of the Th2-associated transcription factors GATA3 and c-maf. GATA3, as well as several other members of the GATA family of transcription factors, has been implicated in the initiation of locus opening for several genes. Indeed ectopic expression of GATA3 is able to facilitate chromatin remodeling of the *IL-4* gene in Th1 cells and allow IL-4 expression (66, 67).

Chromatin remodeling of the *IL-4* gene locus in mast cells

In contrast to T cells, the events that set the stage for chromatin remodeling of the IL-4 locus in mast cells must occur very early in development. Within 3 weeks in culture with IL-3, pluripotent hematopoietic stem cells differentiate into committed mast cell progenitors (BMMC-bone marrow derived mast cells) that express ckit, FcεRI and IL-4 (79). The *IL-4* locus is demethylated at this time and the cells can be activated through the FcεRI to express IL-4 mRNA and protein. This process does not require prior activation or "priming" and is *STAT6* independent: BMMC from $STAT6^{-/-}$ mice also exhibit a demethylated locus within 3 to 4 weeks after initial IL-3 addition and express IL-4 amounts comparable to wild-type levels ((62) and S. K. Lee and M. A. Brown, unpublished observation).

An *IL-4* gene intron regulatory element: role in chromatin accessibility?

The signals that mediate chromatin accessibility of the *IL-4* gene in mast cells have only recently been the subject of intense investigation. Several years ago, DNAse I hypersensitivity assays were performed to identify potential regulatory elements within the murine *IL-4* gene. The most predominant site observed was, surprisingly, within the second intron. This hypersensitive site was only present in IL-4-producing mast cells but not the EL-4 T cell line. Subsequent analyses using conventional enhancer–reporter gene assays revealed that the

intron sequences encompassing this site function as a position- and orientation-independent transcriptional enhancer and can act with both the native IL-4 gene promoter and heterologous promoters (28). Enhancer activity is observed only in IL-4-producing mast cells, but not in EL-4 T cells or the DO11.10 Th2 cell line (J. Hural, M. A. Brown, unpublished observation) and is dependent on sequences forming binding sites for GATA, ets, STAT and a POU-related factor. Electrophoretic mobility shift assays demonstrate that GATA 1, GATA 2 and PU.1, as well as STAT5 specifically associate with these sites in mast cells. Taken together, these data indicate that through its ability to bind factors such as GATA 1/2 and PU.1 that are selectively expressed in mast cells but not T cells, this element exhibits mast cell-specific effects on IL-4 transcription.

The next challenge is to determine the role of this element *in vivo*. Although we originally postulated that this element acts with 5′ promoter proximal elements to enhance transcriptional activation, several observations do not fully support this as its sole role. It was previously shown by Siden et al. that early events in mast cell development are associated with truncated IL-4 transcripts lacking exons I and II (80). Our analysis of these transcripts, present in several unstimulated mast cell lines, revealed that the transcripts initiate at multiple sites within the intron regulatory element at Inr-like promoter elements (D. Powell, M. A. Brown, unpublished observation). The transcripts do not encode an open reading frame but rather contain sequences located within the second intron and extend through exons III and IV of the *IL-4* gene. These IL-4-truncated transcripts are completely analogous to the "sterile" Ig and TCR gene transcripts that initiate within introns near defined enhancer elements. They are observed prior to *VDJ* rearrangement in developing B and T cells, and are considered a hallmark of locus opening (for reviews see (81, 82)). The *TCR* and *Ig* genes undergo differentiation stage-specific recombination events that are dependent, in part, on the presence of *cis*-acting regulatory elements located within the introns. These elements, first identified as transcriptional enhancers in transient transfection assays, influence *VDJ* and *VJ* recombination as well as somatic mutation in the *Ig* genes. Both *in vivo* and *in vitro* studies of the κ and μ chain enhancers confirm their role in influencing accessibility of the *Ig* locus. For example, a transfected μ chain gene construct containing the intron μ enhancer is hypomethylated in pre-B cells, correlating with expression of the heavy chain gene. Deletion of the μ enhancer leads to transcriptional inactivation and *de novo* methylation (83). Similar types of analyses have shown that the κ light chain enhancer also has a role in inducing demethylation of the locus in a lineage- and developmental stage-specific manner (84, 85). Deletion of the *TCR* α and β intron enhancers also negatively affects recombination (86–88).

The similarities between IL-4 and antigen receptor gene regulation prompted us to examine the possibility that the IL-4 intron element also has a role in mediating chromatin accessibility. To test this, two versions of a genomic construct were designed that contain sequences encompassing the *IL-4* chromosomal gene from −800 bp 5′ of the transcription initiation site to ~2 kb beyond exon 4. The constructs were engineered with an oligonucleotide tag inserted just 3′ of the exon IV stop codon to allow the ability to distinguish between mRNA transcripts initiating from the transfected constructs and those from the endogenous gene. The tagged wild-type construct and the deleted intron element construct were transfected into IL-4-producing mast cell line, CFTL15, as well as into the non-IL-4-producing B cell line, M12. The effects of intron deletion on methylation and expression of the IL-4 transgene were then examined.

The methylation state of the transfected sequence was assessed by Southern blot analysis using a pair of enzymes whose recognition site is CCGG: Hpa II, which cannot restrict methylated CpGs, and MspI, which cuts irrespective of the methylation state. We predicted that if the enhancer influences IL-4 gene demethylation, the wild-type construct would be in a demethylated state in mast cells, but the enhancerless gene would be methylated. As shown in *Fig.* 2, this is precisely what we observed. Both the wild-type and enhancerless transgenes are methylated in the B cells, as is the endogenous *IL-4* gene, consistent with the inability of IL-4 to be transcribed in these cells. However, in mast cells, despite the demethylated state of the endogenous *IL-4* gene and the wild-type construct, the enhancerless transgene is methylated. These results were confirmed in cloned transfectant cells as well (*Fig.* 3). These data support the idea that the enhancer influences the chromatin state of the *IL-4* gene locus.

Little is known about the mechanisms through which enhancers act to influence locus opening. However, it is striking that many of the factors that associate with the functional elements of the IL-4 enhancer, including GATA-1/2, PU.1, STAT5 and POU domain family factors, have been implicated in influencing chromatin accessibility at loci encoding other cell-type-specific genes. GATA 1, expressed in myeloid lineage cells, is able to bind to GATA binding sites in nucleosomal DNA and cause extensive and co-operative breakage of the DNA/histone contacts (89). In erythroid cells, mutation of the GATA 1 binding sites in a β globin gene cluster enhancer

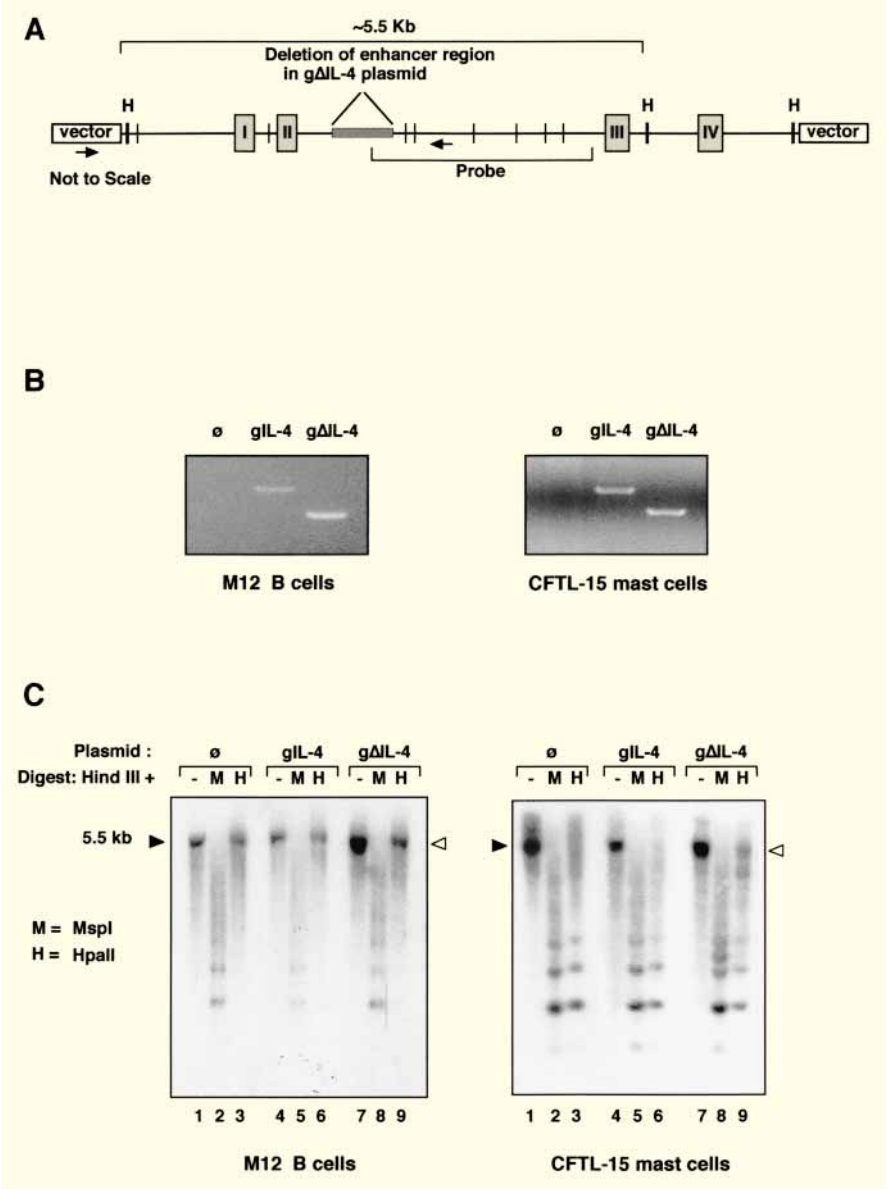

***Fig. 2.* The intron enhancer region is required for the demethylation of the IL-4 locus in mast cells. A) Schematic of IL-4 genomic constructs used in transfection experiments.** *Hind III* sites are indicated by (H). The locations of *Hpa II*/*Msp I* sites within the second intron are designated by (|). Light brackets indicate the *Stu I*/*Eco RI* fragment used as a probe in Southern blot analysis. The approximate position of PCR primers used to verify the integration of the transfected constructs is also shown by arrows. **B) PCR reactions using vector- and gene-specific primers (see *Fig. 2A*) and genomic DNA isolated from M12 B cells and CFTL 15 mast cells that were transfected with the indicated plasmids.** gIL4 designates DNA isolated from cells transfected with the wild-type IL-4 genomic construct; gΔIL-4 designates DNA isolated from cells transfected with the enhancerless IL-4 genomic construct. ∅ designates control cells transfected with the GFP/Neomycin vector alone. **C) Analysis of the methylation status at the IL-4 gene locus in transfectants carrying stably integrated copies of IL-4 genomic constructs with and without the intron enhancer.** Genomic DNAs from M12 B cells and CFTL-15 mast cells were subjected to Southern blot analysis. DNA was digested with either *Hind III* alone (−) (lanes 1, 4, 7) or with *Hind III* followed by either *Msp* I (M) (lanes 2, 5, 8) or *Hpa II* (H) (lanes 3, 6, 9). Blots were probed with a ^{32}P-labeled *StuI-EcoRI* fragment from the second intron of IL-4 (see Fig. 2A above). ∅ designates cells that received the GFP/neomycin vector alone; gIL4 designates DNA isolated from cells transfected with the wild-type IL-4 genomic construct; gΔIL-4 designates DNA isolated from cells transfected with the enhancerless IL-4 genomic construct. Arrow indicates position of Hpa II insensitive Hind III fragment. [Reproduced from experiments published by Hural et al. (74)].

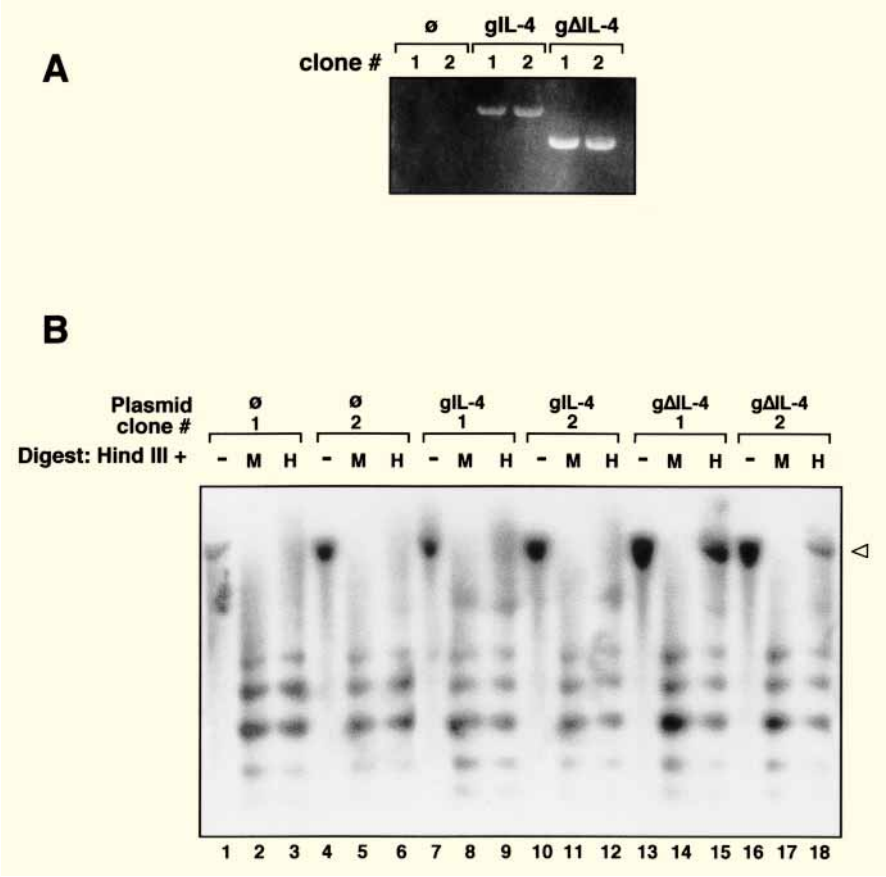

Fig. 3. Clonal populations of mast cell transfectants exhibit the same dependence on the intron regulatory element for demethylation. A) PCR reactions using genomic DNA from cloned CFTL 15 mast cell transfectants. Primers that hybridize with the *pUC18* vector in region 3′ of the intron enhancer sequence are shown in *Fig. 2A*. ∅ designates control cells transfected with the GFP/Neomycin vector alone. gIL4 designates DNA isolated from cells transfected with the wild-type IL-4 genomic construct; gΔIL-4 designates DNA isolated from cells transfected with the enhancerless IL-4 genomic construct. **B) Analysis of the methylation status of the IL-4 gene locus in two different cloned CFTL 15 mast cell transfectants carrying stably integrated copies of IL-4 genomic constructs with and without the intron enhancer.** Genomic DNA from cloned cells was subjected to Southern blot analysis as described above (*Fig. 2C*). *M* denotes samples digested with *Hind III*+*Msp I*. *H* denotes samples digested with *Hind III*+*Hpa II*. – denotes samples digested with *Hind III* alone. Arrow indicates position of *Hpa II* insensitive *Hind III* fragment. [Reproduced from experiments published by Hural et al. (74)].

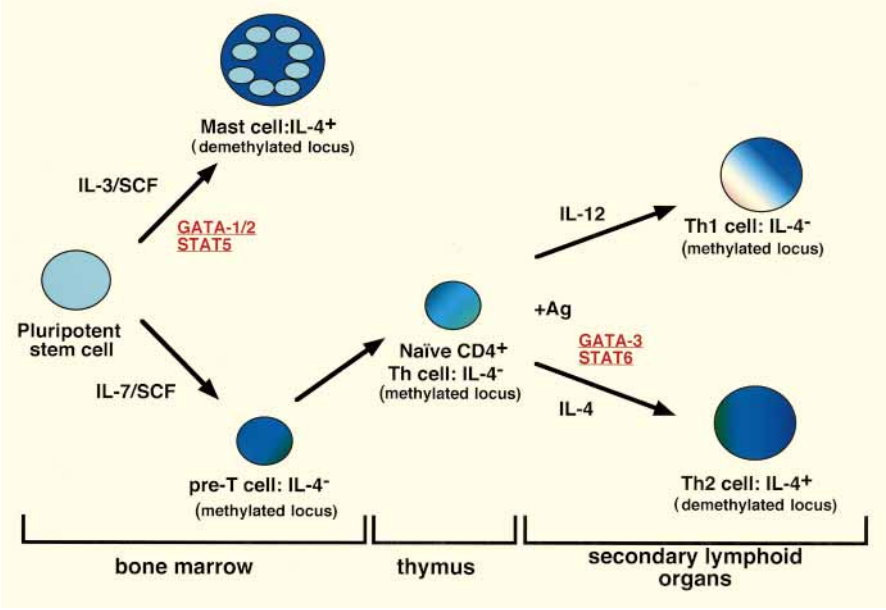

Fig. 4. Th2 and mast cells utilize parallel but distinct pathways for acquiring an IL-4-producing phenotype. Our model proposes that early in mast cell development, the IL-4 gene undergoes chromatin remodeling and acquires an accessible configuration (as measured by the demethylated state and sensitivity to digestion by DNAse I). These events are dependent on the action of GATA-1 and/or GATA-2 and STAT5. In contrast, T cells require additional developmental steps that involve maturation in the thymus and antigen encounter in the presence of IL-4 in secondary lymphoid organs. The activation of STAT6 and the Th2-specific expression of GATA-3 are important influences on the epigenetic changes to the IL-4 locus that lead to IL-4 gene expression. These factors may act at consensus GATA and STAT binding sites within the intron to exert their effects in both T and mast cells.

reduced the accessibility to restriction enzymes (90). These data and others indicate that this factor likely plays an important role in initiating locus-opening events. PU.1, a factor whose expression is limited to B cells, macrophages, neutrophils and mast cells (69, 91–93) binds to plasmids containing the Ig μ enhancer (91, 94). Binding by PU.1 does not alter the nucleosomal array that assembles around the transfected plasmid, but does increase the restriction enzyme accessibility within the enhancer (94). In addition, ectopically expressed PU.1 co-operates with other ets family members to induce Iμ sterile transcripts and to increase chromatin sensitivity to restriction enzyme digestion in stably transfected NIH 3T3 and pro-T cells. Accumulating evidence has shown that in addition to STAT6, other STAT family members are able to interact with transcriptional co-activators such as CBP/p300 and PCAF, proteins that express intrinsic histone acetyltransferase (HAT) activity (76, 95–98). It has been proposed that through their interaction with these co-activators, transcription factors direct this HAT activity to specific gene regulatory regions. STAT5, in particular, activated through IL-7 signaling, can interact with p300/CBP through an adapter protein termed Nmi (96) and has been shown to regulate the accessibility of the TCRγ locus (99, 100).

The established roles for these IL-4 intron binding factors in chromatin opening are consistent with the idea that they play similar roles in modifying *IL-4* gene chromatin in IL-4-producing cells. Further support for this idea comes from experiments which assessed the consequences of single mutations in the GATA, ets and STAT sites within the IL-4 transgene. Mutant GATA transfectants were unable to achieve a completely demethylated locus. The STAT and ets mutations also resulted in only partial demethylation, but the effect was considerably less marked than that observed in the mutant GATA transfectants (74).

Preliminary studies of expression from the transfected IL-4 gene confirm that this region is essential for maximal and importantly, appropriate IL-4 expression. As predicted based on earlier studies in mice, the −800 bp region cannot direct levels of activated transcription equivalent to that observed from the endogenous gene, indicating that sequences outside this region are also important. However, a mutation in the GATA binding site completely obliterates the ability of the transgene to be transcribed. Surprisingly, mutation of the PU.1 binding site results in constitutive expression of high levels of IL-4 mRNA independent of any activation signals (M. Kwan, D. Powell, M. A. Brown, manuscript submitted). This result suggests that PU.1 acts as a repressor, a previously documented activity of this protein (101, 102), to block significant expression in unstimulated cells. We are currently investigating whether these proteins interact with regions of the *IL-4* gene outside the intron.

Parallel but distinct pathways for *IL-4* gene regulation in T and mast cells?

It is still unclear whether the intron element mediates mast cell-specific effects on *IL-4* gene expression. As described above, GATA-1/2 and PU.1 are not expressed in T cells, and we have been unable to demonstrate conventional enhancer activity in Th2-derived cell lines (J. Hural, M. Brown, manuscript in preparation), supporting the idea that this element acts as a mast cell-specific regulatory element. However, the second intron is also demethylated during the course of T-cell differentiation (73); Agarwal & Rao recently demonstrated that the appearance of intron DNase I hypersensitive sites are associated with the development of an IL-4-producing phenotype in Th2 cells (78). Of note, these sites appear to be in close proximity to, but spatially distinct from, those identified in mast cells. These findings raise the possibility that despite its lack of enhancer activity, the second intron is essential for regulating IL-4 transcription in both cell lineages. In T cells, it may only be involved in processes that mediate chromatin remodeling. Whether the same sequences mediate this effect remains to be determined. We propose that related, but distinct factors may act on this element in T cells and serve such a function. In mast cells, GATA-1/2 and STAT5 serve analogous roles in mast cell development and acquisition of an accessible *IL-4* gene locus as GATA-3 and STAT6 play in Th2 development (57, 65). Thus, just as distinct cell-type specific factors regulate transcriptional activation of the *IL-4* gene, different developmental pathways leading to mast cell versus T-cell development may necessitate the use of distinct subsets of factors and sites for locus opening (*Fig.* 4). The apparent commonality of mechanisms that operate on such diverse gene systems such as the *Ig*, *TCR* and *IL-4* genes to regulate accessibility suggests that similar regulatory events occur at other gene loci. Indeed, these data may necessitate the development of a new paradigm for the *in vivo* function of many other gene regulatory elements, first defined as transcriptional enhancers, located in introns or elsewhere.

Conclusions

There is increasing evidence that in addition to causing the pathologic manifestations of allergic disease, mast cells, through their ability to express IL-4 and other immunomodu-

latory cytokines, can influence the generation of a protective response. The identification of factors that control developmental- and activation-induced events leading to IL-4 production is only a first step towards understanding how to manipulate IL-4 in a cell-specific and site-specific manner. Many issues remain to be examined. The external signals that regulate these processes and activate the relevant nuclear factors are still undefined. Because mast cells are difficult to isolate *in situ*, current studies are dependent on the use of long-term lines or BMMC, and the relationship of BMMCs to fully differentiated tissue mast cell populations is unclear. Thus, the IL-4-producing phenotype of fully differentiated mast cells *in vivo* is uncertain. It may be that the ability to express specific mediators is different, depending on the local microenvironment in which a mast cell completes its differentiation. It has been proposed that *IL-4* and other closely linked genes such as *IL-13* and *IL-5* are co-ordinately regulated. Thus, it will also be important to determine whether regulatory elements such as the one defined within the IL-4 intron as well as a conserved non-coding sequence designated CNS-1, located between *IL-4* and *IL-13* (103), have regulatory influences on other genes within this cytokine gene cluster.

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