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Immunology

PU.1 and GATA: Components of a mast cell-specific interleukin 4 intronic enhancer

(transcription factors/cytokine/gene regulation)

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ABSTRACT **Interleukin 4 (IL-4), a critical immunoregulatory cytokine, is produced 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-bp *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. Although mutation of the SP1 sites had no effect on enhancer function, 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.**

The role of mast cells as the primary immune effector cells in IgE-dependent immediate hypersensitivity responses is well characterized. Upon activation through the high-affinity Fcε receptor, mast cells degranulate and release several different types of preformed and newly synthesized biological mediators initiating a variety of events that lead to inflammation (1–3). Evidence has recently accumulated demonstrating that activated mast cells are also a major source of several cytokines, including interleukins 1, 3, 4, 6, and tumor necrosis factor α (3, 4). These findings indicate an additional role for mast cells in processes such as late-phase inflammatory responses, angiogenesis, and wound healing.

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 (5). IL-4 has a unique function in regulating IgE production by B cells and is a mast cell growth factor. 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, 3).

We previously identified an enhancer element in the second intron of the murine IL-4 gene that appears to be mast cell-specific (6). 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 cell-specific activity of this IL-4 intronic enhancer.

MATERIALS AND METHODS

Cell Lines. The transformed mast cell lines ABFTL-3, MMC34, and P815 that express IL-4 mRNA constitutively; the nontransformed line CFTL 12; EL-4 T cells; and WEHI 231 B cells have been described (6), as were the GA15 T-cell hybridoma (7) and CH12 LX 4550 B-lymphoma used in these studies (8). CFTL 12 and EL-4 T cells were stimulated with ionomycin and phorbol 12-myristate 13-acetate, respectively, as described (6). GA15 T cells were stimulated with 2C11, an anti-CD3 specific monoclonal antibody, as described (7).

Reporter Gene Constructs. Details of the derivation of 683/SV40 chloramphenicol acetyltransferase (CAT) have been described (6). (Note that in the original description, this construct was called 670/SV40 CAT. Upon sequencing, the enhancer element was found to contain 683 bp 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, were linearized at one end of the enhancer element with *Xba* I and treated for various times with BAL-31 exonuclease (Pharmacia). BAL-31-treated samples were treated with T4 polymerase, and the vector was religated with T4 ligase. The extent of deletions was determined by DNA sequencing (9). The numbering of nucleotides within the 683-bp fragment is based on designation of the first base of the 5′ *Bgl* II recognition site as number 1.

Site-Directed Mutagenesis. Mutations within the SP1, GATA, and ets consensus binding sites (see Fig. 2) were introduced into the IL-4 intronic sequence containing 255–683 bp (demonstrates full enhancer activity relative to the 683-bp fragment) using the oligonucleotide-directed *in vitro* mutagenesis system version 2.1 (Amersham). All mutations were verified by sequencing. The mutant forms of the oligonucleotides used in the mutagenesis reactions were synthesized by using an Applied Bioscience oligonucleotide synthesizer and are as follows (altered nucleotides are under-

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Abbreviations: IL-4, interleukin 4; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility-shift assay.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U07869).

lined and in boldface type:

SP1 mutant M_A bp 410–387, 5′-CAAATGGAG**CA**GC**T**GGGGGCAGGG-3′;
SP1 mutant M_B bp 431–409, 5′-CACATCTGTC**GGA**TCCCACTACA-3′;
GATA mutant M1 bp 454–430, 5′-GAGAAAATGCA**TCTAG**AGCCTCTCA-3′;
ets mutant M1 bp 520–496, 5′-CCATGAAAACA**TTC**ACTGAAATGCA-3′.

Transfection and CAT Assays. ABFTL-3 cells (10^7) were transfected by electroporation with 6.73 pmol (20–23 μg) of the various CAT reporter plasmids. Cells were harvested 48 hr later, and equal amounts of cell extracts were assayed for CAT activity by using a scintillation diffusion assay as described (6).

Electrophoretic Mobility-Shift Assays (EMSAs). Nuclear extracts were isolated, and EMSAs were done as described (10). In some experiments, 1 μl of antiserum or preimmune serum was added to the binding reaction before the addition of the probe and incubated for 15 min at room temperature. Both GATA-1 monoclonal antibody and GATA-2 antiserum were from Stuart Orkin (Children's Hospital, Boston). The PU.1-9794 antiserum (11) was from David Kabat (Oregon Health Sciences University) and PU.1 peptide antiserum 1297 (12) was provided by Richard Maki (La Jolla Cancer Research Foundation).

RESULTS

Two Distinct Subregions Contribute to IL-4 Enhancer Activity. We previously identified a 683-bp *Bgl* II fragment within the second intron of the IL-4 gene that exhibits mast cell-specific enhancer activity (6). To define the precise cis-acting elements that regulate this enhancer, deletions from both ends of the *Bgl* II fragment were generated. The effects of these deletions on the enhancement of simian virus 40 promoter-driven CAT gene expression in ABFTL-3 mast cells were determined. As shown in Fig. 1, three 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. A PCR-generated sequence from 353 to 554 enhanced CAT gene expression at levels equivalent to the entire 683-bp fragment (data not shown), confirming these results.

The partial activity of constructs containing further deletions suggests that the enhancer is composed of at least two elements that can act independently to enhance transcription. There is a 50–60% decrease in activity when comparing the response of the 5′ deletion constructs Δp2 and Δp3, indicating that sequences between 346 and 444 are critical for full enhancer activity. This region has been designated E1 (Fig. 1*A*). The residual activity of Δp4 (40–50% 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 50–60% decrease in activity confirms the presence of this second region, designated E2 (Fig. 1*B*). Furthermore, the contribution of E1 was confirmed by the decrease in activity of Δp7 when compared with 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 Fig. 2. Analysis of this sequence revealed that there are several sequences that demonstrate identity to previously defined cis-acting regulatory elements. 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) (13) and a GATA-binding site (438–443) (14) were identified within E1 (Fig. 2). An ets binding site (15) is located within the E2 region at position 503–510.

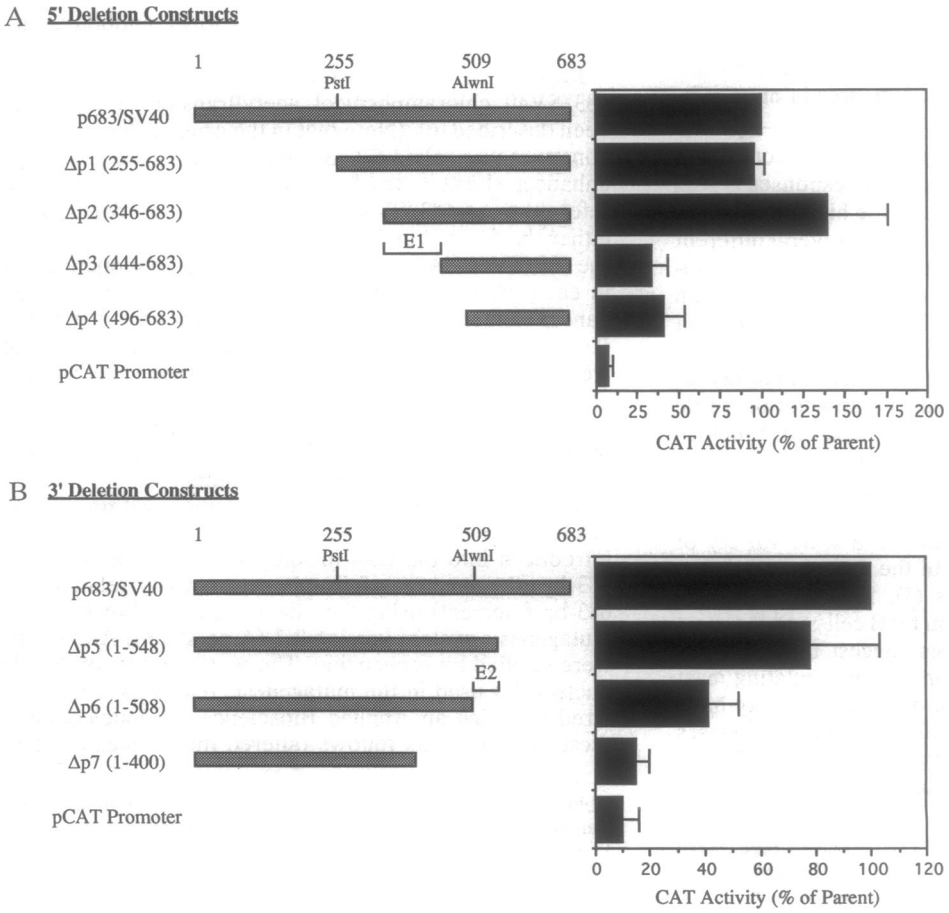

FIG. 1. Effect of 5′ and 3′ end deletions on an IL-4 intronic enhancer activity. CAT reporter gene constructs containing deletions from either the 5′ (*A*) or 3′ (*B*) ends of the 683-bp *Bgl* II intronic fragment were generated using BAL-31 nuclease. These were compared to the parent construct (683/SV40) for the ability to enhance simian virus 40 promoter-driven CAT gene expression in transient transfection assays. The relative enhancer activity of the truncated constructs is expressed as the mean percentage of activity of the parent vector (set at 100) ± SE of three to five experiments. The numbering of coordinates is based on designating the first base of the 5′ *Bgl* II site as 1.

FIG. 2. Sequence of the functionally relevant region of the IL-4 intronic enhancer (bp 348–548). The consensus binding sites for SP1, GATA, and ets are shown in boldface 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 (13), for GATA is WGATAR (14), and for ets is (G/C)AGGA(A/T) (15).

The IL-4 GATA and ets-Like Binding Sites Are Critical for Enhancer Activity. To assess the possible contribution of SP1, GATA, and ets sites in IL-4 enhancer activity, single or multiple site-directed mutations that disrupt each of the core-binding sites were introduced into Δp1 (255–683), a construct that exhibits full enhancer activity (see Fig. 1*A*). Mutation of a single SP-1 site (data not shown) or both SP-1 sites together (dm-SP1) has no effect on enhancer function, whereas mutation of either the GATA (mt-GATA) or ets site (mt-ets) reduces enhancer activity by 50–60% (Fig. 3). 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.

The GATA and ets Sites Are the Target of Specific DNA–Protein Interactions. EMSAs were done by using ABFTL-3 nuclear extracts and labeled oligonucleotide probes containing either the IL-4 GATA or ets binding site (Fig. 4) to assess DNA–protein interactions at these sites. Two complexes form with the GATA site probe (designated A and B, lane 1). A single protein–DNA complex is observed with the ets site probe (lane 5). The specificity of these interactions was confirmed in EMSA experiments in which a 100-fold excess of unlabeled competitor DNA was added to the binding reactions. The detection of both GATA complexes is inhibited by 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

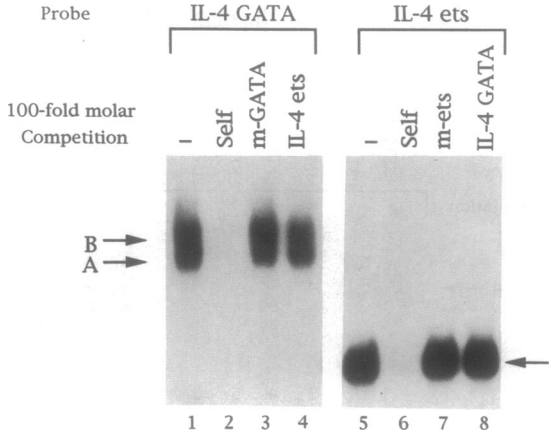

FIG. 4. Both the IL-4 GATA and ets sites form specific DNA–protein complexes. EMSAs were done by using 0.1 ng of ^{32}P-labeled GATA (lanes 1–4) or ets (lanes 5–8) DNA probes incubated with 5 μg of ABFTL-3 nuclear extract in the absence (–) or presence of 10 ng (100-fold molar excess) of the indicated competitor DNAs. 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 Fig. 3. The two complexes formed with the GATA probe are indicated by arrows A and B. The single ets complex is indicated by an arrow.

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. To explore the possibility that differential binding of transcription factors to the GATA and/or ets sites accounts for the cell type specificity of this enhancer, 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 Fig. 5*A*, 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 those observed with ABFTL-3 extracts (lanes 1, 3–5). P815 extracts form only the lower mobility complex. Extracts from T- (lanes 6–9) and B-cell lines (lanes 10 and 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 with the other cell types examined.

Cell-specific differences in binding activity to the IL-4 ets site probe were also observed (Fig. 5*B*). All mast cell extracts

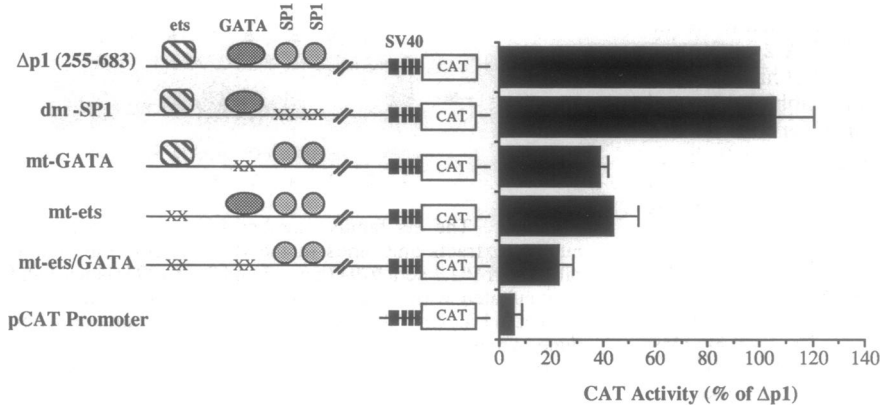

FIG. 3. Effect of mutations within the core recognition region of the IL-4 intronic SP1, GATA, and ets binding sequences on enhancer activity. Single or double mutations were introduced within the SP1, GATA, and/or the ets site in Δp1. Xs represent altered sites. The relative enhancer activity of the mutant constructs is expressed as a mean percentage of the activity of Δp1 (set at 100) ± SE of three to five experiments.

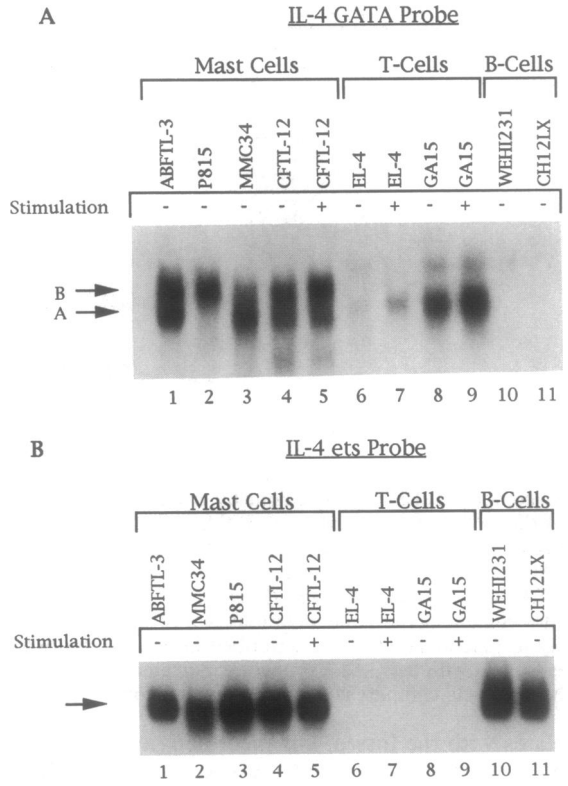

FIG. 5. Differential binding to the IL-4 GATA and ets sequence correlates with enhancer function. EMSAs were done by using nuclear extracts from unstimulated (−) or stimulated (+) cells of mast cell (lanes 1–5), T-cell (lanes 6–9), and B-cell (lanes 10 and 11) lineage. CFTL 12 mast cells were stimulated with 0.75 μM ionomycin for 30 min. EL-4 cells were stimulated with phorbol 12-myristate 13-acetate at 10 ng/ml. GA15 T cells were stimulated with 2C11 (1 μg/ml for 12 hr). (*A*) EMSA with the IL-4 GATA oligonucleotide probe. (*B*) EMSA with the IL-4 ets oligonucleotide probe.

form a single specific complex of the same mobility as the ABFTL-3 DNA–protein interaction. However, no binding activity is observed in T-cell extracts. This is noteworthy because T cells express several ets family members (16). 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 (17). GATA-1 and GATA-2 are expressed in erythrocytes, megakaryocytes, and mast cells but not in T cells. To determine the presence of GATA-1 and GATA-2 in the mast cell IL-4 GATA-site complexes, antibodies that specifically react with each factor were added to standard EMSA binding reactions. A GATA-1-specific monoclonal antibody blocks complex A formation and has no effect on complex B (Fig. 6, lane 2). GATA-2 antiserum interferes with complex B formation (lane 3) but has no effect on GATA-1 complex formation. Preimmune rabbit serum and antiserum to an ets family member 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 (18). These factors recognize small differences in the ets binding sites within the regulatory regions of several genes. Two observations indicated that the ets family member PU.1 is associated with the IL-4 ets site. The intronic ets-like site

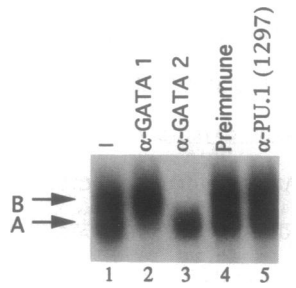

FIG. 6. GATA-1 and GATA-2 are present in ABFTL-3 cells and bind to the IL-4 intronic GATA site. One microliter of anti-GATA-1 (lane 2) or anti-GATA-2 (lane 3) was preincubated with 5 μg of ABFTL-3 nuclear extract in binding buffer. One microliter of either preimmune rabbit serum or ets-specific antiserum (anti-PU.1) was used as negative control (lanes 4 and 5, respectively).

shares 10 of 11 bp of identity with the simian virus 40 PU.1-binding site (19). 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 (20–22). To determine whether PU.1 associates with the IL-4 ets sequence, mobility shift assays were done with the IL-4 ets site probe using ABFTL-3 nuclear extracts in the presence of two different PU.1-specific antibodies (Fig. 7). An extract from WEHI 231 cells, a B-cell line previously shown to express PU.1 (23), was used as a positive control. PU.1-9794 antiserum, from animals immunized with the whole PU.1 protein (11), 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 (12) also inhibits complex formation with the IL-4 ets probe (lanes 3 and 8). Neither preimmune serum nor 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 activated T cells and cells of the mast cell/basophil lineage (5). However, the cell-surface receptors that trigger inducible expression of IL-4 by each cell type are distinct. Thus, the regulatory mechanisms that control IL-4 expression within these cell types are likely to differ. 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 (6). In this study we have demonstrated that at least two functionally independent subregions, designated E1 and E2, contribute to this

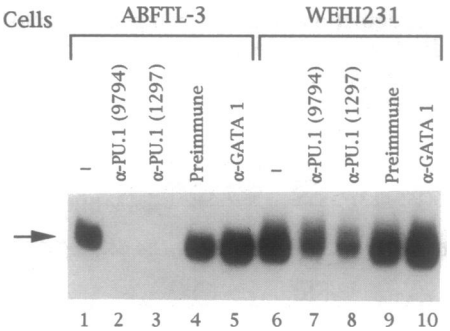

FIG. 7. The ets family member PU.1 is present in mast cell nuclear extracts and binds to the IL-4 ets site. One microliter of rabbit anti-PU.1 sera 9794 (lanes 2 and 7) or 1297 (lanes 3 and 8) was preincubated with 5 μg of either ABFTL-3 or WEHI 231 nuclear extract in binding buffer. One microliter of preimmune rabbit serum (lanes 4 and 9) or anti-GATA 1 (lanes 5 and 10) was used as negative control. (*A*) EMSA with ABFTL-3 mast cell nuclear extract. (*B*) EMSA with WEHI 231 B-cell nuclear extract.

activity. Sites within these subregions interact *in vitro* with the previously described transcription factors GATA-1 and GATA-2 and PU.1, respectively. Mutation of both the GATA and ets sites together does not eliminate all enhancer activity (Fig. 3), indicating that additional regions may contribute to enhancer function. DNase I footprint analysis demonstrated a candidate region between E1 and E2 (bp 440–470) that specifically interacts with proteins (G.H., unpublished observation).

The activity of the enhancer correlates with the differential expression in T and mast cells of proteins that associate with the GATA and ets sites. This observation provides a molecular explanation for the mast cell-specific activity of the enhancer. PU.1 and GATA-1 and -2, present in mast cells, but not the T-cell lines we examined, exhibit highly restricted, cell-specific expression (17, 20–22) and regulate the cell-specific expression of other genes. PU.1, previously defined as a macrophage and B-cell-specific transcription factor (20), is a regulator of the myeloid-specific CD11b promoter and the B-cell-specific J chain gene promoter (11, 24). GATA-1 and GATA-2 regulate the mast cell-specific expression of the carboxypeptidase A gene (25).

T cells contain specific GATA-binding activity; however, the enhancer fails to function (6). T-cell lines, including EL-4, express another member of the GATA family, GATA-3 (26). This transcription factor binds to the WGATAR consensus site with a high affinity and regulates the transcription of several T-cell receptor genes (27). 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 differential transactivation requirements for T- and mast cell-specific GATA transcription factors that account for the lack of activity in T cells.

It is notable that although T lymphocytes express other ets family members (16), we are also unable to detect any T-cell protein binding to the IL-4 intronic ets site. 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 (15, 28, 29).

Questions regarding the physiologic role of the intronic enhancer and its activity in human mast cells also remain unanswered. Although first defined in murine-transformed mast cell lines, the enhancer functions in unstimulated, nontransformed mast cells (6). This activity correlates with the protein–DNA binding data that demonstrate 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 (30, 31). 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.

Comparison of the murine intronic enhancer sequence with the equivalent region in the human IL-4 gene (32) demonstrates there is complete identity within the GATA and ets core binding sites as well as the closely flanking sequences (G.H., 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: (*i*) differential expression of transcription factors in mast versus T cells and (*ii*) 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.

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