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Expression of the IL-5 gene is restricted to the Th2 subset of helper T cells. We have previously defined four cis-regulatory elements of the IL-5 promoter responding to PMA and cAMP in EL-4 cells. We now report that the 1.2-kb region of the IL-5 promoter directs expression of the IL-5 gene in a Th2 clone but not a Th1 clone, indicating that transcription from the IL-5 promoter is Th2 specific. For the functioning of the IL-5 promoter in a Th2 clone, IL-5C and IL-5CLE0 were critical. IL-5CLE0 interacted with both constitutive and inducible nuclear factors (designated NFIL-5CLE0), which existed in both Th1 and Th2 clones, whereas IL-5C interacted with a constitutive nuclear factor (designated NFIL-5C), which was found only in Th2 but not in Th1 clones. Th2 specificity of NFIL-5C was also confirmed using in vitro-differentiated Th1 and Th2 cells derived from TCR-transgenic mice. The sequence for NFIL-5C binding bears homology with GATA-binding sites. The NFIL-5C complex was supershifted by an anti-GATA-3 Ab and inhibited by an oligonucleotide containing GATA-binding sites. We showed preferential expression of GATA-3 in Th2 cells. Finally, we demonstrated that in vitro-translated GATA-3 bound to IL-5C and overexpression of GATA-3 augmented stimulation-dependent IL-5 promoter activity in EL-4 cells. Taken together, our results provide evidence that GATA-related factors may be involved in Th2-specific expression of the IL-5 gene.


Interleukin 5 is a key regulator of growth and differentiation of eosinophils and thus is implicated in the pathogenesis of eosinophil-associated disorders such as asthma and allergy (1). The critical role of IL-5 in the pathogenesis of allergic lung disease has been demonstrated using a mouse asthma model with IL-5-deficient mice (2). Thus, understanding the control mechanisms for IL-5 production could provide important targets for the treatment of allergic disease. IL-5 is produced primarily by T cells upon activation, and in mice, the expression of IL-5 is restricted to the Th2 subset of helper T cells (3).

The two types of major immune responses, cellular and humoral immunity, are differentially regulated by distinct sets of cytokines derived from Th1 and Th2 cells; Th1 cells produce IL-2, IFN-γ, and TNF-β and promote cellular immunity, whereas Th2 cells produce IL-4, IL-5, IL-6, and IL-10, help in B cell functioning, and also are associated with allergic responses (4–7). Thus, a proper balance of Th1 and Th2 responses to a particular Ag or pathogen is crucial, and dysregulation of Th1 and Th2 responses is often related to disease states (6, 7). Evidence indicates that Th1 and Th2 cells differentiate from a common precursor (8, 9). Furthermore, it has been shown that IL-12 and IL-4 play critical roles in Th1 and Th2 differentiation, respectively (10). Recently, selective expression of the IL-12Rβ2 subunit on Th1 cells during Th1/Th2 differentiation has been demonstrated, and the regulatory role of IL-4 and IFN-γ on this process has been shown (11–13).

The molecular mechanisms underlying differential cytokine production in Th1 and Th2 cells remain unclear. Differences in signal transduction pathways between Th1 and Th2 cells could result in differential cytokine production. Several reports have shown that PGE2, which elevates the intracellular cAMP level, has different effects on cytokine production in Th1 and Th2 cells, i.e., it inhibits cytokine production from Th1 cells but not from Th2 cells (14, 15). There is also an indication that the cAMP level in Th1 cells is higher than in Th1 cells (16). Other reports suggest that TCR-mediated calcium influx is selectively impaired in Th2 cells (17, 18). Selective expression of transactivators or repressors in Th1 and Th2 cells could also lead to differential cytokine production. However, the possibility of the existence of selective repressors has been refuted by a study showing that somatic cell fusion of a Th1 and a Th2 clone gives rise to a cell type that produces both Th1- and Th2-type cytokines (19).

To date, differential expression of two cytokine genes, IL-2 and IL-4, has been shown to be regulated by differential transcriptional activity of their promoters in Th1 and Th2 cells (20, 21). The 300-base pair (bp) region of the IL-2 promoter can mediate Th1-specific expression of the gene (20, 21). However, no cis-regulatory element within this region has been shown to be involved in differential regulation of the IL-2 gene in Th1 and Th2 cells. Although circumstantial evidence suggests that differential regulation of NF-κB may be involved in Th1-specific expression of the IL-2 gene (21), no direct evidence has been provided. Extensive studies have focused on the IL-4 gene, and it has been shown that the control region of Th2-specific gene activity resides within the

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3 Abbreviations used in this paper: bp, base pair(s); EMSA, electrophoretic mobility shift assay; kb, kilobase; Bt(cAMP), N6,O2′-dibutyryl cAMP; HPRT, hypoxanthine-guanine phosphoribosyltransferase.
MECHANISMS FOR TH2-SPECIFIC EXPRESSION OF THE IL-5 GENE

proximal 800-bp IL-4 promoter (21, 22). Furthermore, the region spanning −60 to −35 has been shown to confer Th2-specific promoter activity (20). This region has also been shown to interact with Th2-specific transcription factor c-Maf, thereby controlling tissue-specific expression of the IL-4 (19).

On the other hand, the molecular mechanisms by which Th1 and Th2 cells differentially express the IL-5 gene remain largely unknown. Using EL-4 cells, we have found that cAMP is essential for PMA-dependent expression of the IL-5 gene, while it almost completely inhibits that of the IL-2 gene (23). cAMP exerts its differential effects on the expression of the IL-2 and IL-5 genes through the promoter regions. We have previously identified four cis-regulatory elements (designated IL-5A, IL-5P, IL-5C, and IL-5CLEO) (24) that are necessary for full activity of the IL-5 promoter in response to PMA and cAMP signals.

In this study, we examined the cis-regulatory elements and nuclear factors that account for Th2-specific expression of the IL-5 gene, using Th1 and Th2 clones as well as in vitro-grown Th1 and Th2 cells from OVA-specific TCR-transgenic mice.

Materials and Methods

Reagents

N6,O2-dibutyryl cAMP (Bt2cAMP) was purchased from Sigma Chemical Co. (St. Louis, MO) and used at final concentrations of 1 mM. PMA, A23187, and ionomycin were purchased from Calbiochem (La Jolla, CA) and were used at final concentrations of 50 ng/ml, 0.5 μM, and 1 μM, respectively.

Th clones

Stimulation and maintenance of Th clones were as described (25). D10.G4.1 (from Dr. C. Janeway, Yale University, New Haven, CT), a conalbumin-specific Th2 clone derived from AKR/J mice, and HDK1 (26), a keyhole limpet hemocyanin-specific Th2 clone derived from BALB/c mice, were maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 200 μM mouse rIL-2, and 10% FCS. Th clones were stimulated every 2 wk with irradiated splenic APCs from mice of appropriate haplotype and specific Ags and cultured for 2 wk before preparation of RNA and nuclear extracts.

Cytokines, Abs for cytokines, and peptide

Mouse rIL-4 was from Dr. S. Menon (DNAX Research Institute). Mouse rIL-12 was obtained from PharMingen (San Diego, CA). Purified rat anti-IL-4 mAbs (C17.8) were as described (28). The antigenic OVA peptide from chicken OVA (OVA323–339) was synthesized on an Applied Biosystems model 430 peptide synthesizer (Foster City, CA).

Transgenic CD4+ T cells

Preparation and culture of polarized Th1 and Th2 populations from TCR-transgenic mice (D011.10 TCR-αβ) on a BALB/c genetic background (29) were as previously described (30). CD4+ T cells were stimulated weekly with OVA-peptide (OVA257–264, 0.3 μM) presented by irradiated BALB/c splenic APCs in the presence of IL-12 (10 ng/ml) and anti-IL-4 (11B11, 10 μg/ml), to polarize toward the Th1 phenotype, or IL-4 (10 ng/ml) and anti-IL-12 (C17.8, 10 μg/ml), to polarize toward the Th2 phenotype.

Plasmids

pmlSLuc(1.2), which contains the mouse IL-5 promoter (−1174 to +33) fused to the luciferase gene, as well as the promoterless control pUC001Luc have been described (23). pmol2–321Luc containing the mouse IL-2 promoter (−327 to +46) fused to the luciferase gene is described in Tsu-ruta et al. (31), and the linker-scanning mutants of the IL-5 promoter, L59/3993, L510397, L569/63, and L575/51, are as previously described (24). The reference plasmid pRSV-LacZ in which the β-galactosidase gene is under the control of the Rous sarcoma virus LTR was provided by Dr. A. Tsuboi (National Institute for Basic Biology, Okazaki, Japan). The plasmid mc5b8 was transcribed from the T7 promoter and translated in a wheat germ lysate using a TriT-Coupled Transcription/Translation Kit (Promega) according to the manufacturer’s instruction.

Transfection into Th clones and EL-4 cells

Th clones were transfected with plasmid DNA by electroporation 4 days after allogenic stimulation. The cells were washed once with serum-free RPMI 1640, then resuspended at 2.5 × 105 cells/ml. Cell suspensions (0.4 ml, 1 × 105 cells) were incubated with 10 μg of reporter constructs and 1 μg of pRSV-LacZ for 10 min at room temperature and transferred to 0.4 cm electroporation cuvettes (Bio-Rad, Richmond, CA). The cells were electrophorated using a Bio-Rad Gene Pulser at 310 V and 960 μF and allowed to recover for 10 min at room temperature. After culture for 24 h in the fresh medium, the cells were either unstimulated or stimulated with PMA and A23187 for 16 h and harvested for luciferase as well as β-galactosidase assays.

EL-4 cells (1 × 105 cells) were transfected with 1 μg of reporter plasmid, 4 μg of activator plasmid, and 0.15 μg of pRSV-LacZ by the DEAE-dextran method as described previously (23). At 36 h after transfection, the cells were either unstimulated or stimulated with PMA and ionomycin for 12 h as assayed above.

Antibodies

The mouse monoclonal anti-GATA-3 Ab (34) was provided by Dr. M. Yamamoto (University of Tsukuba, Tsukuba, Japan), and the GATA-4 Ab, GATA-4 (C20), a goat polyclonal Ab, was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RNA extraction and RT-PCR analysis

Total cellular RNA was isolated from T cell clones or Th1 and Th2 cells using a RNasea kit (Qiagen, Chatsworth, CA). One microgram of total RNA was reverse transcribed in a 20 μl reaction, and the cDNA samples were diluted with sterile distilled water. An aliquot of this was used for a PCR reaction using each pair of sense and antisense primers given in Table II. The number of PCR cycles and the amount of cDNA were titrated for each primer set, and then a condition that gave an optimal signal without saturation was chosen. PCR conditions were 94°C for 2 min, followed by: 20 to 30 cycles of 94°C, 30 s; 60 to 65°C, 30 s; 72°C, 30 s. The PCR products were separated on agarose gels, stained with CBBR Green (Molecular Probes, Eugene, Oregon), and were then visualized using ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Preparation of nuclear extracts and EMSAs

Cells were treated for 2 h with various reagents, as described in the figure legends, and nuclear extracts were made as described (35). EMSAs were performed using the double-stranded oligonucleotides given in Table II. Each single-stranded oligonucleotide was purified on a denaturing polyacrylamide gel before annealing. The annealed oligonucleotides were 32P labeled with Klenow fragment and purified on 12% polyacrylamide gels. The DNA-binding reactions were performed at room temperature for 30 min with 2 to 5 μg of nuclear extracts, 0.5 μg of poly(dI-dC), 10 mM HEPES (pH 7.9), 10% glycerol, 1 mM EDTA, 1 mM DTT, 100 mM KCI, and 0.5 ng of probe in a total volume of 10 μl. The samples were resolved on a 5% nondenaturing polyacrylamide gel at 120 V in 0.5× Tris-borate-EDTA buffer, and the results were visualized by autoradiography. Protein content was determined by the Bradford assay with a kit provided by Bio-Rad.

Results

Differential expression of the IL-2 and IL-5 genes in the Th1 clone, HDK1, and Th2 clone, D10.G4.1, cells

The Th1 clone, HDK1, produces IL-2 in response to a T cell activation signal, whereas the Th2 clone, D10.G4.1, produces IL-5 (26). In addition, PMA and calcium ionophore stimulation can induce IL-5 production to the same extent as anti-CD3 stimulation in several Th2 clones, such as D10.G4.1 and CDC35 (25).

To verify exclusive expression of the IL-2 gene by HDK1 cells and of IL-5 by D10.G4.1 cells, we performed sensitive RT-PCR analysis using RNA from unstimulated and stimulated cells (Fig. 1).
The Th1 clone, HDK1, expressed IL-2 but not IL-5 in response to PMA and ionomycin stimulation, whereas the Th2 clone, D10.G4.1, expressed IL-5 and a barely detectable amount of IL-2. We have previously reported that cAMP activated the IL-5 gene synergistically with PMA, whereas it suppressed PMA-induced IL-2 transcription in EL-4 cells (23). There are also other indications suggesting involvement of the cAMP signal in IL-5 production (14, 15, 25, 36, 37). However, HDK1 (Th1) cells did not transcribe the IL-5 gene even in the presence of cAMP, although its inhibitory effect on IL-2 gene expression could be detected. Unlike EL-4 cells in which the cAMP signal is essential for IL-5 expression, D10.G4.1 (Th2) cells expressed a considerable amount of IL-5 with PMA and calcium ionophore stimulation alone, and cAMP had only an augmenting effect.

**Differential activity of the IL-2 and IL-5 promoters in HDK1 and D10.G4.1 cells**

To assess whether the Th2-specific expression of the IL-5 gene is directly associated with the promoter activity, an IL-5 promoter-reporter gene construct was introduced into HDK1 and D10.G4.1 cells by transient transfection, as described in Materials and Methods. An IL-2 promoter construct was also tested in parallel, as a Th1-specific control. We previously showed that the 1.2-kb region of the IL-5 promoter can mediate the transcriptional specificity of their respective genes in Th1 and Th2 cells.

**IL-5C and IL-5CLE0 are critical for promoter activity in D10.G4.1 cells**

The differential ability of D10.G4.1 and HDK1 cells to induce IL-5 promoter activity may result from selective expression of an essential positive factor(s) or a repressor(s) in respective cell types. To address this hypothesis, we investigated possible positive or negative elements in the IL-5 promoter by mutation analysis. We had previously defined four cis-regulatory elements—IL-5A, IL-5P, IL-5C, and IL-5CLE0—of the IL-5 promoter responding to PMA and Bt2cAMP in EL-4 cells (Fig. 3A). To examine whether any of these cis elements was responsible for Th2-specific activation of the IL-5 promoter, four luciferase reporter plasmids, each carrying a mutation in one of the four elements, i.e., IL-5A (LS939/993), IL-5P (LS103/97), IL-5C (LS69/63), and IL-5CLE0 (LS57/59) (Fig. 3A), were transiently transfected into HDK1 and D10.G4.1 cells. Constructs containing a mutation in either IL-5A or IL-5P still retained IL-5 promoter activity, albeit at a reduced level, whereas mutations in either IL-5C or IL-5CLE0 abrogated IL-5 promoter activity in response to PMA and A23187 in D10.G4.1 cells (Fig. 3B). Thus, both IL-5C and IL-5CLE0 are essential for promoter activity in D10.G4.1 cells, while the IL-5A and IL-5P are required for optimal activity. On the other hand, none of these constructs was activated in HDK1 cells, suggesting that cis-acting repressor elements may not reside within these four sites.

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**Table I. Primer sequences used in PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stranda</th>
<th>Sequence (5’ to 3’)</th>
<th>Size of PCR Productb</th>
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<td>TGAAGACCTCACGAGGTCTCTAGGG</td>
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<td></td>
<td>A</td>
<td>GATCATTTACCCAGAATGCTTCTCAAGCAAGAG</td>
<td>211</td>
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<td>CGAGCTCTGGTGGACAAAGCAATGAGACGAAT</td>
<td>214</td>
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<tr>
<td></td>
<td>A</td>
<td>CGTCTCTGCAGAGGAAGGCTCCTCTTTTT</td>
<td>246</td>
</tr>
<tr>
<td>HPRT</td>
<td>S</td>
<td>GAACTGCTCCAGCTGGCAAGGGGGGAGG</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>GGAAGTTGGTACCTGACAGGGGGGGAGG</td>
<td></td>
</tr>
<tr>
<td>GATA-3</td>
<td>S</td>
<td>GCTTTGCTGCTGTTCTCTCTCTCTCTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>GCTTTGCTGCTGTTCTCTCTCTCTCTCT</td>
<td></td>
</tr>
<tr>
<td>IL-12Rβ2</td>
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<td>CGAAAAATTCACTACCGAGCGACTCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>GCTTTGCTGCTGTTCTCTCTCTCTCTCT</td>
<td></td>
</tr>
</tbody>
</table>

a S indicates sense strand; A, antisense strand.

b Numbers in parentheses are references.

c Uppercase nucleotides are derived from the promoter sequence, and lowercase nucleotides indicate overhang sequences, respectively.

d Only the sense strands are depicted.

e Numbers in parentheses are references.

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**Table II. Oligonucleotide probes used in EMSAa**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequenceb</th>
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<td>74-38</td>
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</tr>
<tr>
<td>IL-5</td>
<td>5’-tcaAGTGGACCTCAGAGGAGGTCTCTAGGG-3’</td>
</tr>
<tr>
<td>IL-5CLE0</td>
<td>5’-tcaAGTTGGAATTTCTCTCTCTCTCTAGGA-3’</td>
</tr>
<tr>
<td>NF-κB (31)</td>
<td>5’-ggATCAGAGGATTTCTCTCTCTCTCTCTAGGA-3’</td>
</tr>
<tr>
<td>ToGATA (39)</td>
<td>5’-TTGAGAGATGGCCAGGGGGGAGGGGAGG-3’</td>
</tr>
<tr>
<td>Spl (52)</td>
<td>5’-TTGAGAGATGGCCAGGGGGGAGGGGAGG-3’</td>
</tr>
</tbody>
</table>
Differential binding of nuclear factors to the −74 to −38 region in HDK1 (Th1) and D10.G4.1 (Th2) cells

The essential role of IL-5C and IL-5CLE0 for IL-5 promoter activity suggests that in Th1 cells, the lack of IL-5 promoter activity

FIGURE 1. RT-PCR analysis of IL-2 and IL-5 gene expression in HDK1 and D10.G4.1 cells. HDK1 and D10.G4.1 cells were either unstimulated (−) or stimulated for 6 h with PMA and ionomycin (+) or PMA and ionomycin plus Bt2cAMP (++) Total RNA was prepared, and RT-PCR analysis was performed as described in Materials and Methods. Amplification products corresponding to IL-2, IL-5, and HPRT are indicated. The data are representative of three separate experiments using at least two independent cDNA preparations.

FIGURE 2. Analysis of IL-2 and IL-5 promoter activity in the Th1 and Th2 cells. HDK1 and D10.G4.1 cells were transiently transfected with control plasmid pUC00Luc, IL-5 promoter construct pmIL5Luc(1.2), or IL-2 promoter construct pmoIL-2-321Luc and then were split into three groups. After a 24-h incubation, cells were either unstimulated (open bars) or stimulated for 16 h with PMA and A23187 (solid bars), then harvested for luciferase assays. The activity of pGL2-promoter without stimulation was assigned a value of 1.0; promoter activity was expressed as relative fold increase over pGL2-promoter activity after normalization for β-galactosidase activity of pRSV-LacZ. The results are typical of at least three independent experiments.

FIGURE 3. Mutation analysis of the 5′-upstream region of the IL-5 promoter. A, Schematic representation of the murine IL-5 promoter. The 1.2-kb promoter region and the positions of the functional cis elements, IL-5A, IL-5P, IL-5C, and IL-5CLE0, are shown as solid boxes. The nucleotide sequence of the IL-5 gene from −1174 to +33 is depicted. The IL-5 promoter constructs LS939/933, LS103/97, LS69/63, and LS57/51 contain mutations on the positions of IL-5A, IL-5P, IL-5C, and IL-5CLE0, respectively, in the context of the 1.2-kb promoter. B, Effect of mutation on the cis-regulatory elements of the IL-5 promoter on the promoter activity. HDK1 and D10.G4.1 cells were transiently transfected with the IL-2 promoter-luciferase construct and the IL-5 promoter-luciferase constructs depicted in A. After a 24-h incubation, cells were either untreated (open bars) or stimulated for 16 h with PMA and A23187 (solid bars), then harvested for luciferase assays. The activity of pUC00Luc without stimulation was assigned a value of 1.0; promoter activity was expressed as relative fold increase over pUC00Luc activity after normalization for β-galactosidase activity of pRSV-LacZ. The results are typical of at least three independent experiments.
may be due to the absence of factors operating on these sites. Since, in many cases, cell type-specific gene expression is controlled by cell type-specific transcription factors, we examined whether IL-5C and IL-5CLE0 interact with DNA-binding proteins in a Th2-specific manner. Since no clear boundary between these two elements has been identified, we initially used the −74 to −38 region, which includes both IL-5C and IL-5CLE0 (−74 to −38, Fig. 4A), as a probe in EMSA. Nuclear extracts from D10.G4.1 cells stimulated with PMA and ionomycin formed four principal complexes with the 74–38 probe (I, II, III, and IV, Fig. 4B, lane 4). Comparing the electrophoretic profile of unstimulated (lane 3) and stimulated (lane 4) D10.G4.1 nuclear extracts, the amount of complex II and III was increased in stimulated D10.G4.1 cells (lane 4), whereas the amount of complex I and IV was not changed upon stimulation. In HDK1 cells, which did not express the IL-5 gene (Fig. 4A, lane 3), whereas complex IV was not detected in HDK1 cell extracts, raising the possibility that a component(s) of the complex is Th2 specific. Other minor complexes with different mobility could be detected, but their appearance was variable depending on the different nuclear extract preparations. NF-κB binding, as a control, was detected in both cell types (lanes 5–8). Consistent with previous reports, the ratio of the p65/50 form to p50/50 is higher in HDK1 cells than D10.G4.1 cells (21).

NFIL-5C and NFIL-5CLE0 bind to IL-5C and IL-5CLE0, respectively

To check the sequence specificity for each complex, segments of the sequence of the 74–38 probe were mutated and tested for their effects on complex formation (Fig. 5A). All four complexes were effectively inhibited by the addition of unlabeled wild-type 74–38 oligonucleotide (Fig. 5B, lane 2). Interestingly, addition of mutIL-5C, which contains a 3-bp substitution on the IL-5C element, inhibited complex I, II, and III but not IV (lane 3). On the other hand, addition of mutIL-5CLE0, which contains a 3-bp substitution on IL-5CLE0, inhibited complex IV but not I, II, and III (lane 4). The control NF-κB oligonucleotide did not affect any of these complexes (lane 5). Thus, it appeared that complex I, II, and III bound to IL-5CLE0, whereas complex IV bound to IL-5C. To further confirm this conclusion, we next used two smaller overlapping oligonucleotides for the binding competition assay. As expected, the IL-5C oligonucleotide (Fig. 5A, residues −74 to −54) was able to inhibit complex IV as effectively as the 74–38 oligonucleotide, but not complex I, II, and III (Fig. 5C, lane 3), whereas the IL-5CLE0 oligonucleotide (Fig. 5A, residues −61 to −36) inhibited complex I, II, and III but not complex IV (lane 4). A direct binding assay also showed that the IL-5 probe formed a complex.
with a nuclear factor(s) (designated NFIL-5C) that migrated with mobility similar to complex IV (lane 5), whereas the IL-5CLE0 probe formed complexes with nuclear factors (designated NFIL-5CLE0) that migrated with mobility similar to complex I, II, and III (lane 6). The slight difference in mobility between the complex IV and NFIL-5C may be caused by the different configuration of protein-DNA complexes between the probes, since a similar difference was also observed in EMSA using in vitro-translated GATA-3 proteins (data not shown). The difference in mobility of the protein-DNA complex, depending on the location of the binding site on DNA, has been demonstrated in a GATA protein (38).

NFIL-5C-binding activity is Th2 cell specific

We next examined the Th1 and Th2 specificity of NFIL-5C by EMSAs using nuclear extracts from HDK1 and D10.G4.1 cells either unstimulated or stimulated with PMA and ionomycin. The NFIL-5C complex appeared constitutively only in nuclear extracts from D10.G4.1 cells but not from HDK1 cells (Fig. 6A). To confirm that these were not artifacts instilled by long-term culture, we further verified the Th2 specificity of NFIL-5C using freshly generated Th1 and Th2 cells. Th1 and Th2 cells were generated in vitro from CD4+ T cells derived from OVA-specific transgenic mice as described in Materials and Methods. EMSAs using nuclear extracts from Th1 and Th2 cells revealed that the appearance of the NFIL-5C complex is restricted to Th2 cells (Fig. 6B, lanes 4 and 5) and absent from Th1 cells (lanes 2 and 3) regardless of stimulation. Sp1 binding was detected in both cell types (lanes 6–9). These results demonstrated that NFIL-5C is a Th2-specific factor(s) and also strongly suggested that NFIL-5C plays a role in regulating the expression of the IL-5 gene in a Th2 cell-specific manner.

NFIL-5C contains a GATA protein

To identify specific bases critical for NFIL-5C complex formation, serial 3-bp mutations were introduced in the IL-5C site (Fig. 7A) and tested for effects on complex formation by binding competition analysis. Addition of Cm1, Cm2, and IL-5CLE0 oligonucleotides failed to inhibit binding (Fig. 7B, lanes 4–7 and 12), whereas addition of Cm3 and Cm4 oligonucleotides inhibited binding as effectively as the wild-type IL-5C oligonucleotide (Fig. 7B, lanes 8–11).
7B, compare lanes 2 and 3 with lanes 8–11). Similarly, the oligonucleotide containing the mutations at position −73 to −71 inhibited the binding (data not shown). These results indicate that residues −69 through −63 were essential for NFIL-5C binding. It is important to note that these sequences contain overlapping binding sites for GATA proteins (Fig. 7A).

Thus, we examined whether NFIL-5C is related to GATA proteins by competition EMSAs with an oligonucleotide (TrGATA) containing a GATA-3 binding site from the human T cell receptor α-chain gene enhancer (39). NFIL-5C complex formation was inhibited by addition of excess TrGATA as well as IL-5C oligonucleotides (Fig. 8A, lanes 2 and 3), but not by the IL-5CLE0 oligonucleotide (lane 4). Conversely, the unlabeled IL-5C oligonucleotide was able to inhibit the formation of the DNA-protein complex when the labeled TrGATA probe was used (lane 7).

It has been shown that the oligonucleotides containing IL-5C interact with GATA-3 in EL-4 cells (40) and GATA-4 in ATL-16T cells (41), a T cell line derived from an adult T cell leukemia patient. We examined whether the NFIL-5C complex contains GATA-3 or GATA-4 by supershift EMSAs (Fig. 8B). The NFIL-5C complex from both D10.G4.1 and in vitro-generated Th2 cell nuclear extracts was supershifted by an Ab specific for GATA-3 (34) (lanes 2 and 5), but not by an Ab raised against GATA-4 (lanes 3 and 6). Another GATA-4 antiserum that does not cross-react with GATA-1, -2, or -3 (42) did not supershift the NFIL-5C complex (data not shown). Similar results were obtained with the TrGATA probe (data not shown). These Abs did not supershift the NF-κB complexes (lanes 8 and 9).

Differential expression of GATA-3 in Th1 and Th2 cells
Supershift EMSA results indicated that the Th2-specific nuclear factor NFIL-5C is GATA-3 or a related protein(s). Thus, we next examined whether GATA-3 is differentially expressed in Th1 and Th2 cells. RT-PCR analysis was performed using RNA from unstimulated and stimulated Th1 and Th2 clones as well as in vitro-generated TCR-transgenic Th1 and Th2 cells (Fig. 9A). GATA-3 expression was detected only in the Th2 clone, D10.G4.1 cells, but not in the Th1 clone, HDK1 cells. To compare the level of GATA-3 transcripts in HDK1 and D10.G4.1 cells, we titrated the amount of cDNA in PCR reactions. As shown in Figure 9B, D10.G4.1 cells express a significantly higher level of GATA-3 than HDK1 cells, while both cells express a comparable level of HPRT transcripts. This differential expression of GATA-3 was also observed in Ag-specific Th2 and Th1 cells obtained from the TCR-transgenic mice (Fig. 9A). These data are in keeping with recent findings from Zheng and Flavell (43). In contrast, IL-12Rβ2 transcripts were detected in HDK1 and Th1 cells but not in D10.G4.1 and Th2 cells, as previously reported (12, 13). As expected, IL-5 expression was restricted to D10.G4.1 and Th2 cells.

GATA-3 binds the IL-5C element and directly regulates the IL-5 promoter via the IL-5C element
We next asked whether GATA-3 directly regulates IL-5 promoter activity through the IL-5C element. First, we determined whether recombinant GATA-3 can bind to IL-5C. We prepared murine GATA-3 proteins by in vitro transcription and translation using a wheat germ system and tested their ability to bind IL-5C. The IL-5C probe formed a DNA-protein complex with wheat germ extracts from in vitro transcription and translation reactions programmed with recombinant GATA-3, but not with those programmed with the control vector (Fig. 10A, lanes 1 and 2). The complex formation was inhibited by the wild-type (WT) IL-5C oligonucleotide (lane 3) but not by the Cm1 oligonucleotide (lane 4), which failed to bind NFIL-5C (Fig. 7B). Thus, we concluded that GATA-3 binds to the IL-5C site in a sequence-specific manner.
We next investigated whether GATA-3 can directly activate the IL-5 promoter. EL-4 cells were cotransfected with an expression plasmid containing the murine GATA-3 cDNA and the IL-5 promoter construct. Overexpression of GATA-3 per se, without stimulation, did not activate the IL-5 promoter. However, overexpression of GATA-3 augmented stimulation-dependent IL-5 promoter activity as much as sixfold as compared with cotransfection of control plasmid pME18S (Fig. 10B). The transactivation of the IL-5 promoter by GATA-3 required the IL-5C element, since GATA-3 did not transactivate LS69/63, which contains a mutation in IL-5C. Taken together with the results from the in vitro binding assay, these results suggest that GATA-3 regulates the IL-5 promoter by direct interaction with the IL-5C site, but is not sufficient by itself to activate the IL-5 promoter.

Discussion

The molecular mechanisms by which Th1 and Th2 cells produce different cytokines remain largely unknown. Extensive studies have focused on the IL-4 gene (20–22, 44, 45). The proto-oncogene c-maf, which has been shown to be selectively expressed in Th2 cells (19), transactivates the IL-4 promoter through the Maf recognition element (MARE). However, the Th2-specific association of c-Maf with this element was not demonstrated. More recently, Zheng and Flavell have shown that GATA-3 is selectively expressed in Th2 cells (43) and is involved in Th2 cytokine gene expression. However, they did not define any functional cis-regulatory elements of the Th2 cytokine genes with which GATA-3 might interact.

Here, we provide strong evidence that Th2-specific expression of the IL-5 gene is controlled by the interaction of a Th2-specific transcription factor with the critical cis element of the gene. Using Th1 clone HDK1 and Th2 clone D10.G4.1 as a model system, we explored the molecular mechanisms underlying differential expression of the IL-5 gene in Th1 and Th2 cells. First, we confirmed, by sensitive RT-PCR assays, that induction of IL-5 gene transcription was restricted to D10.G4.1 cells and that cell type-specific expression of the IL-5 gene was directly associated with the inducibility
of the promoter in D10.G4.1 cells but not HDK1 cells. We further demonstrated that IL-5C and IL-5CLE0 were critical for the function of the IL-5 promoter in D10.G4.1 cells. Moreover, IL-5C interacts with the D10.G4.1 cell-specific nuclear factor (NFIL-5C), which is related to GATA-3. Finally, we show that GATA-3 is preferentially expressed in D10.G4.1 (Th2) cells. Importantly, these findings were not limited to HDK1 and D10.G4.1 cells. We further confirmed our results using freshly generated TCR-transgenic Th1 and Th2 cells (30), which strongly suggests that this is not a phenomenon restricted to Th clones carried long-term in vitro.

Sequence similarity between the binding sequence of NFIL-5C and GATA protein consensus and cross-competition for binding between IL-5C and TcrGATA suggest that the NFIL-5C complex contains GATA proteins. Involvement of GATA proteins in IL-5 gene transcription through IL-5C has also been shown in other systems using tumor cell lines (40, 41). GATA-3 seems to be the major component of the NFIL-5C complex, since most of the complex was supershifted by anti-GATA-3 Ab. This notion is in good agreement with our RT-PCR results showing that the expression profile of GATA-3 in these cells was closely correlated with the appearance of the NFIL-5C complex. Indeed, recombinant GATA-3 could bind to IL-5C specifically and activated the IL-5 promoter via the IL-5C element. These results, together with the overlapping expression profile of the GATA-3 and the IL-5 genes, suggest that GATA-3 may control Th2-specific expression of the IL-5 gene through IL-5C. GATA proteins are a group of transcription factors with a C4 zinc finger DNA-binding domain that recognizes the consensus sequence A/TGATA/G/A as well as some related sequences (46). GATA-3 is preferentially expressed in the T cell hemopoietic lineage and plays an important role in T cell development (47). Functional GATA sites have been identified on the enhancers of several T cell-specific genes including the TCR (48).

Our results are in agreement with the recent report by Zheng and Flavell that identified GATA-3 as a gene selectively expressed in Th2 cells by cDNA subtraction between in vitro-generated Th1 and Th2 cells (43). Their antisense as well as transgenic results suggest clear involvement of GATA-3 in the expression of Th2 cytokine genes such as IL-4, IL-6, IL-10, and IL-13, but the effect of a reduced level of GATA-3 on IL-5 expression was minimal in the antisense GATA-3-expressing D10 cells. However, our results suggest that IL-5C is absolutely required for the IL-5 promoter activity in D10 cells. The results from Zheng and Flavell (43) indicate that the difference in the level of GATA-3 in the control D10 cells and the antisense GATA-3-expressing D10 cells is not as dramatic as that seen in Th1 and Th2 clones. Their results also clearly showed increased expression of the IL-5 gene, albeit to a lesser extent than IL-4, IL-6, or IL-10, in IL-12-driven Th1 cells obtained from GATA-3-transgenic mice. Zheng and Flavell (43) also demonstrated that TCR expression levels were not altered by a reduced expression of GATA-3 and that the extent of inhibition was different among Th2 cytokines. Thus, the GATA site of each of these genes may have a different affinity for GATA-3, and the range of the GATA sites being occupied may depend on the level of GATA-3 expression. In this respect, it is interesting to note that the IL-5C sequence comprises the inverted overlapping GATA-3-binding sites with high affinity (3’ part) and intermediate affinity (5’ part) (49). It has been reported that double GATA sites often have higher affinity for GATA proteins (50). In addition, chromatin accessibility and interaction with other factors could also affect preferential association of GATA-3 with each target site in vivo. Alternatively, the NFIL-5C complex may consist of other factors closely related but not identical to GATA-3. Further in vivo and in vitro experiments will be required to clarify this question.

Mutational analysis indicated that not only IL-5C but also IL-5CLE0 was indispensable for IL-5 promoter activity. In contrast to NFIL-5C, NFIL-5CLE0 was induced by stimulation in both HDK1 and D10.G4.1 cells. However, we cannot rule out the possibility that the components and/or modification status of the NFIL-5CLE0 complex may not be exactly the same between Th1 and Th2 cells, resulting in differential IL-5 gene transcription. Interestingly, Naora et al. reported that the oligonucleotide containing IL-5CLE0 interacted with an inducible nuclear factor present in D10.G4.1 cells but not in HDK1 cells, using their specific stimulation and binding conditions (51). Thus, it may be possible to distinguish the Th2-specific component(s), if any, within the NFIL-5CLE0 complex under the conditions they used. Further biochemical analysis will be required to clarify the components of NFIL-5CLE0 and its role in Th2-specific expression of IL-5.

Since both IL-5C and IL-5CLE0 are essential for the functioning of the IL-5 promoter, cells that have both NFIL-5C and NFIL-5CLE0 can support promoter activation (Fig. 11). Unstimulated Th1 cells in which neither IL-5C nor IL-5CLE0 is occupied are unable to induce IL-5 promoter activity. Upon stimulation, NFIL-5CLE0 complex formation is induced, but is still insufficient for IL-5 promoter activation. Similarly, occupation of IL-5C by NFIL-5C in unstimulated Th2 cells is not sufficient for promoter activity. Further occupation of IL-5CLE0 by NFIL-5CLE0 after stimulation is needed to trigger promoter activation. This notion is in good agreement with the expression profile of the GATA-3 and IL-5 as well as the result from cotransfection of GATA-3, indicating that both GATA-3 and stimulation-dependent factors are required to activate the IL-5 gene. Currently, we do not know how NFIL-5C and NFIL-5CLE0 cooperate to activate the IL-5 promoter. It is possible that binding of both sites may trigger the recruitment of additional factors, such as coactivator and/or transcription initiation machinery, to turn on the IL-5 promoter. Further studies will be required to address this hypothesis.

It is important to note that the cis-regulatory elements that are essential for the IL-5 promoter to respond to PMA and cAMP in EL-4 cells (24) are also critical in D10.G4.1 cells. The sequences within the IL-5CLE0 and IL-5C are highly conserved between human and mouse IL-5 genes, and these elements are also important for the functioning of the human promoter when assessed in EL-4 cells (24). Thus, it appears that the principal regulatory elements of the IL-5 gene are conserved between the human and mouse.
Taken together, our studies dissect the critical regulatory mechanisms for IL-5 expression, which may provide not only an insight into the pathogenesis of IL-5-associated allergic diseases but also the potential for alternative approaches to therapy. Furthermore, this system will provide a useful tool for studying the mechanisms for differential regulation of cytokine genes in Th1 and Th2 cells.

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