Activation of the Interleukin-5 Promoter by cAMP in Murine EL-4 Cells Requires the GATA-3 and CLE0 Elements*

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Interleukin-5 (IL-5) plays a central role in the growth and differentiation of eosinophils and contributes to several disease states including asthma. Accumulating evidence suggests a role for cAMP as an immunomodulator; agents that increase intracellular cAMP levels have been shown to inhibit production of cytokines predominantly produced by Th1 helper (Th) 1 cells such as IL-2 and interferon-γ (IFN-γ). In contrast, the production of IL-5, predominantly produced by Th2 cells, is actually enhanced by these agents. In this report, we have performed transient transfection experiments with IL-5 promoter-reporter gene constructs, DNase I footprinting assays, and electrophoretic mobility shift assays to investigate the key regulatory regions necessary for activation of the IL-5 promoter by dibutyryl cAMP and phorbol esters in the mouse thymoma line EL-4. Taken together, our data demonstrate the critical importance of two sequences within the IL-5 5'-flanking region for activation by these agents in EL-4 cells: one, a highly conserved 15-base pair element present in genes expressed by Th2 cells, called the conserved lymphokine element 0 (CLE0; located between −53 and 0 in the IL-5 promoter), and the other, two overlapping binding sites for the transcription factor GATA-3 (but not GATA-4) between −70 and −59. Taken together, our data suggest that activation via the unique sequence combination GATA/CLE0 results in selective expression of the IL-5 gene in response to elevated levels of intracellular cAMP.

Interleukin-5 (IL-5) is the key cytokine that regulates the biological functions of eosinophils and contributes to several human disease states, including asthma (1, 2). In both atopic and non-atopic asthma, elevated IL-5 has been detected in peripheral blood and the airways (3–5). There is considerable interest in the identification of the transcriptional mechanisms controlling the synthesis of this cytokine. IL-5 is predominantly produced by activated T helper 2 (Th2) lymphocytes, although mast cells and eosinophils have been also shown to produce this cytokine (2, 6–8). Murine T helper 2 cells are classified into two distinct subsets (Th1 and Th2) on the basis of their patterns of lymphokine secretion. Whereas IL-5 gene expression is restricted to the Th2 subset of CD4+ cells, which also express IL-4 and IL-10 (but not IL-2 or interferon-γ, which are produced by Th1 cells), GM-CSF is produced by both Th1 and Th2 cells (9, 10). In contrast to cytokines such as IL-2, IL-5 is strongly induced by factors that raise intracellular cAMP levels, such as IL-1α, prostaglandin E2, and forskolin (11–14). In murine Schistosoma mansoni infection, vasoactive intestinal peptide released from eosinophils induces adenylate cyclase in T cells via vasoactive intestinal peptide receptors, resulting in IL-5 production (15). In transient transfection assays, dibutyryl cAMP (Bt2cAMP)-induced activation of the IL-5 promoter was mimicked by transfection of an expression plasmid encoding the catalytic subunit of protein kinase A, suggesting that cAMP stimulates IL-5 transcription via the protein kinase A signaling pathway (16). The observation that cAMP increases the expression of cytokines such as IL-5, while suppressing that of other cytokines such as IL-2 and interferon-γ (IFN-γ), suggests a possible regulatory role for this second messenger (9, 10, 13, 17, 18).

The IL-5 promoter contains potential binding sites for multiple transcription factors including NF-AT, AP-1, Oct, and Elf-1 (19, 20). The AP-1- and Elf-1-binding sites together constitute an element called the consensus lymphokine element 0 (CLE0) found in cytokine genes including IL-3, IL-4, IL-5, and granulocyte/macrophage-colony stimulating factor (GM-CSF) (21–23). This element plays a crucial role in the regulation of the GM-CSF gene (22, 24, 25).

In these studies, we sought to define precisely the cis-activating elements that regulate inducible murine IL-5 transcription in EL-4 cells in response to Bt2cAMP and PMA, both of which are required for optimal stimulation of the IL-5 promoter in these cells (16, 26). Our data suggest that activation of the IL-5 promoter by Bt2cAMP and PMA in EL-4 cells requires sequences within the CLE0 element and also a region located between −70 and −59 that binds the transcription factor GATA-3. We speculate that activation via this unique sequence combination confers the specificity needed for selective expression of the IL-5 gene in response to elevated levels of intracellular cAMP.

MATERIALS AND METHODS

Cell Culture—EL-4 cells were obtained from American Type Culture Collection (ATCC) and maintained in suspension culture in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% horse serum at 37 °C with 5% CO2.

Plasmid Preparation (Deletion Constructs)—A murine IL-5 promoter plasmid, p4k-μUC18, was a kind gift from Dr. Honjo. The luciferase
reporter plasmid pXPI was obtained from ATCC. Deletion constructs were prepared by polymerase chain reaction, using the following primers for the upstream sequence: p545L5Luc (ATGGATCCCTCGCCTTTATTAGG), p76L5Luc (ATGGAATTCCTCGCCTTTATTAGG), and the primer p91L5Luc (ATGGAATTCCTCGCCTTTATTAGG) for the downstream sequence. These primers incorporated a restriction site for BamHI and HindIII in the upstream and downstream sequences, respectively. These polymerase chain reaction products were cloned into pXPI. Another deletion construct p118L5Luc was prepared by digesting p545L5Luc with Hpal and HindIII and ligating back into pXPI.

Site-directed mutants were generated following the method of Kunkel using a kit from Bio-Rad (27). All constructs were confirmed by DNA sequencing.

Transfection—EL-4 cells were washed once in serum-free Dulbecco's modified Eagles' medium and resuspended in the same medium. ~1 × 10^5 cells in 0.8 ml were combined with approximately 40 μg of DNA (5 μg of reporter plasmid, 1-2 μg of cytomegalovirus-β-galactosidase plasmid, and approximately 33 μg of the carrier plasmid pGEM7Z), and electroporation was carried out with the Gene Pulser (Bio-Rad) at 0.25 kV, 960 microfarads. The cells were then immediately returned to 4.8 ml of the same growth medium and incubated at 37 °C, 5% CO_2, 16 h after transfection, the cells were split into two aliquots. The first aliquot received no stimulation, while the second aliquot was stimulated with 25 ng/ml PMA and 1 mM Bt2cAMP for 8 h.

Luciferase and β-Galactosidase Assays—The luciferase assay was performed essentially according to manufacturer's instructions as described previously (28). Briefly, cells were harvested 8 h after stimulation and washed twice in phosphate-buffered saline. They were then lysed with 100 μl of lysis buffer for 10–15 min, and debris was removed by centrifugation. 20 μl of supernatant was combined with 100 μl of luciferase assay reagent and immediately analyzed in a Berthold luminometer. In order to adjust luciferase values for relative transfection efficiency, β-galactosidase assays were performed as described previously (29). Since the basal adjusted relative light units (~250–300) were slightly higher than the background readings of reagent blanks (~150–200), the background readings were not subtracted from each experimental value to calculate the fold-induction.

DNase I Footprinting Assays—Nuclear extracts were prepared from ~1–1.5 × 10^6 EL-4 cells. Cells were washed in ice-cold phosphate-buffered saline and incubated for 10 min on ice in 200 μl of buffer A containing 10 mM Hepes, pH 7.9, 1.5 mM MgCl_2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM EDTA. After incubation, a 0.1 volume of 1% Nonidet P-40 was added and the lysates were immediately centrifuged at low speed (~350 × g) at 4 °C for 2 min. The supernatants were discarded, and the pelletted nuclei were resuspended in 50 μl of a lysis buffer containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl_2, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. After incubation for 15 min at 4 °C with vigorous shaking, the lysed nuclei were centrifuged at 16,000 × g for 10 min to clear the debris. The supernatants were aliquoted, snap-frozen, and stored at −70 °C until further use.

A fragment containing sequences between ~168 and ~24 of the IL-5 promoter was used for footprinting assays using techniques previously described (30). Labelled fragment (−30 fmol) was incubated at room temperature for 15 min in 100 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 5% glycerol, 0.05% Nonidet P-40, 1 mM MgCl_2, 0.2% polyvinyl alcohol, 10 μg/ml poly(dI)-poly(dC), in the presence or absence of nuclear extract prepared from EL-4 cells. At the end of the incubation, 100 μl of a salt mixture (10 mM MgCl_2, 5 mM CaCl_2) was added to stop reaction. DNase I (Worthington) was added to a final concentration of 0.5 or 2.5 units/ml to tubes with or without nuclear extracts, and digestion was carried out for 1 min at room temperature. The reactions were stopped with 200 μl of stop buffer containing 0.2 M NaCl, 0.04 M EDTA, 1% SDS, 125 μg/ml tRNA, and 100 μg/ml proteinase K. The samples were extracted with a mixture of phenol/chloroform, and the RNA was precipitated with ethanol and electrophoresed on 8% polyacrylamide, 8.3 × urea gels. For accurate reading of the footprints, a T = C sequencing reaction of the labeled strand was electrophoresed in parallel.

Electrophoretic Mobility Shift Assays—The probes in the EMSAs were three 20-bp double-stranded oligonucleotides containing sequences between i) −57 and −34 (containing the CLE0 element), ii) −93 and −73, and iii) −93 and −73. The probe for the CLE0 element containing probe, the binding reaction contained 10 μM Hepes, pH 7.9, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM MgCl_2, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml poly(dI)-poly(dC), and 200 μg/ml bovine serum albumin. The buffer composition was the same for the other two probes, except that the MgCl_2 was added at 1 mM for the CLE0 probe. The competitor oligonucleotides were added at a 100- or 200-fold molar excess. Antibodies to the Jun, NF-κB, and GATA-3 proteins were purchased from Santa Cruz Biotechnology, while the anti-Fos antibody was obtained from Oncogene Science. The anti-GATA-3 antibody was raised against a glutathione S-transferase (GST)-human GATA-3 (amino acids 1–264) fusion protein (31). Anti-GATA-4 antibody, kindly provided by Dr. David Wilson, was rabbit anti-mouse GATA-4 antisemum that does not cross-react with GATA-1, -2, or -3 (32).

RESULTS

Activation of the IL-5 Promoter Requires the AP-1-Like Site and Additional Upstream Sequences—Fig. 1A depicts the IL-5 promoter and potential transcription factor binding sites based on sequence homology. Considerable information exists regarding the role played by NF-AT in the transcriptional regulation of IL-2 (33). CLE1 represents a “conserved lymphokine element” found in the 5′ upstream sequence of a variety of cytokine promoters, including GM-CSF, IL-3, and IL-5 (21, 22). The role it plays in the transcriptional regulation of these genes is essentially unknown. The CLE0 element appears to represent a composite element consisting of a consensus sequence for binding of Εts family proteins, more specifically Eif-1, and an AP-1-like binding site, which in the GM-CSF gene appears to bind both JunB and c-Fos (25). Both the Eif-1- and the AP-1-binding sites are crucial for transcriptional activation of the GM-CSF promoter (24, 25). The IL-5 CLE0 element differs from the GM-CSF CLE0 element by only 1 base pair in the AP-1-like region (Fig. 1C).

In order to determine precisely the region in the IL-5 promoter responsible for transcriptional regulation, EL-4 cells were transiently transfected with luciferase reporter plasmids incorporating IL-5 gene sequences ranging from 545 to 66 bp upstream from the transcription start site (Fig. 1B). An 8-h stimulation with Bt2cAMP and PMA caused an 8–10-fold increase in transcriptional activity in the 545-bp construct. While the 168-, 118-, and 91-bp constructs also gave a similar response, ~40–50% activity was induced in the construct containing 76 bp of sequence upstream of the transcription start site. However, the 5′-flanking sequence deleted to –66 was unresponsive to the stimuli (Fig. 1C).

To further define the key cis-regulatory elements in the IL-5 promoter, transient transfection experiments using site-directed mutants were performed (Fig. 1C). Mutations in NF-AT and CLE1 decreased transcriptional activity modestly (Fig. 1B). It has been well established that activation of NF-AT occurs via a Ca_2+—dependent mechanism (33). Previous work indicated that elevations in [Ca_2+] will play a minimum role in modulating the transcriptional regulation of the IL-5 gene, suggesting that NF-AT probably does not play a major role in the transcriptional activity of IL-5 (34–36). It is important to note, however, that activation of the NF-AT element in EL-4 cells by PMA alone, in the absence of a Ca_2+-activated pathway, is probably due to a constitutive increase in [Ca_2+], in EL-4 cells (37). In these studies, the preservation of near full activity in the deletion construct p91L5Luc, compared to the 545-bp construct, further supports the absence of a critical contribution by NF-AT-activated pathways in the activation of IL-5 gene transcription (34–36).

Fig. 1B shows that a mutation in the AP-1 sequence within the CLE0 element eliminated activity of the IL-5 promoter. This observation indicated that transcription factors binding to the AP-1 or overlapping sequences within the CLE0 element are critical (but not sufficient) for transcriptional activity of IL-5 in response to Bt2cAMP and PMA.
Stimulation of EL-4 Cells with Bt2cAMP and PMA Protects the IL-5 Promoter from Digestion with DNase I—In order to identify potential sites for transcription factor binding in response to stimulation by Bt2cAMP and PMA, footprinting experiments utilizing DNase I digestion were performed (Fig. 2). Footprints that spanned almost the entire sequence from −88 to −41 were detected (Fig. 2). They included one clearly distinguishable footprint spanning nucleotides −68 to −41 and a second less clear footprint across the region −88 to −77 (Fig. 2). While the footprint between −53 and −39 was due to proteins binding to the CLE0 element, the region between −88 and −54 appeared to be binding sequences for as yet uncharacterized proteins. Thus, regions of the murine IL-5 promoter that showed functional activity in the transient transfection experiments also demonstrated DNase I protection, presumably reflecting the binding of transcription factors important for the transcriptional activation of the IL-5 promoter in response to Bt2cAMP and PMA. Although the footprints were more marked with nuclear extracts prepared from Bt2cAMP + PMA-activated cells, nuclear proteins isolated from unstimulated cells also showed some binding activity corresponding to these regions (Fig. 2). A third footprint between −30 and −16 included the TATA box and adjacent sequences. No footprints were identified in the region corresponding to the NF-AT and CLE1 elements.

Bt2cAMP and PMA Induce Specific Transcription Factor Binding to the Murine IL-5 Promoter CLE0 Element—In order to identify specific transcription factor binding to the murine IL-5 promoter, EMSAs were performed. Using an oligonucleotide containing the IL-5 CLE0 element as probe, at least three complexes were readily detected with nuclear extracts from stimulated EL-4 cells (Fig. 3A, lane 2). Although the ratio of protein:DNA used in these experiments was less than that used in the DNase I assays, nuclear extracts prepared from unstimulated cells also displayed some DNA binding activity (Fig. 3A, lane 1). An identical unlabeled oligonucleotide, used in 100-fold molar excess competed for binding (lane 3); in contrast, an unlabeled oligonucleotide containing the consensus sequence for AP-1 competed partially only for formation of complexes I and II but not at all for complex III (lane 4). A mutation within the IL-5-AP-1-like sequence totally abolished the ability of an oligonucleotide to compete for formation of any complex (lane 6). Taken together, the results depicted in lanes 4 and 6 suggest that the AP-1 sequence within the IL-5 CLE0 site is critical for binding but proteins that bind to this element probably cannot be fully accounted for by factors which can bind a consensus AP-1 sequence. An oligonucleotide incorporating a mutation within the AP-1-like site within the IL-5 CLE0 element competed completely for binding, suggesting that the AP-1-binding sequence is not involved in binding of transcription factors to the IL-5 CLE0 element in these cells (lane 5).

The AP-1-like Site in the IL-5 CLE0 Element Binds Transcription Factors Consistent with JunB and JunD—In order to characterize the proteins that bound to the IL-5 CLE0 element, antisera against the AP-1 family and NF-κB proteins were used in the same EMSA (Fig. 3A, lanes 7–12). An antiserum that recognizes all members of the Jun family significantly affected formation of complexes I and II and only reduced complex III to the level seen in uninduced cells (lane 7). When antisera against the individual Jun proteins were used, anti-c-Jun inhibited the formation of either complex I or II minimally (lane 8). Antiserum against JunB and JunD appreciably inhibited the binding activity in complexes I and II (lanes 9 and 10). An antiserum that recognizes all members of the Fos family.
plexes (complex III was similar to the effect of the anti-Jun antiserum only weakly affected formation of complex I. The effect on family abolished most of the binding activity in complex II, but the Region spanning nucleotides −93 to −54—the region of the IL-5 promoter upstream of the CLE0 element—contained critical functional activity and corresponded to a footprint within the region from −41 to −68 and a second weaker footprint between −88 and −77 (the former includes the AP-1-like sequence; Figs. 1C and 2). We used a radiolabeled 40-bp oligonucleotide containing the entire sequence between −93 and −54 (but excluding the CLE0 element) in the IL-5 promoter to investigate specific binding of proteins to this region in EMSAs (Fig. 3B). Two DNA-protein complexes were readily detected using this particular probe. The faster migrating complex, which could be detected using extracts from both unstimulated and stimulated cells, was clearly nonspecific in nature (Fig. 3B). However, the complex displaying slower electrophoretic mobility was less readily detectable when nuclear extracts were prepared from unstimulated cells, but its formation was significantly induced following stimulation of the cells for 8 h with Bt2cAMP and PMA (Fig. 3B, lane 1). A third complex (see Figs. 4 and 5), which was also largely inducible, was detected with excess nuclear extract. The complex shown in Fig. 3B represented a sequence-specific DNA binding activity because it was efficiently competed for by excess of the same unlabeled competitor oligonucleotide (Fig. 3B, lane 3) but not by oligonucleotides corresponding to the IL-5 CLE0 or the IL-5 NF-AT/CLE1 sites (lanes 4 and 7, respectively) or by the nonspecific competitors containing consensus AP-1 and NF-κB sites (lanes 5 and 6, respectively). Antibodies against the Jun and Fos family members also did not interfere with binding, further demonstrating that the transcription factor(s) binding to this region in the IL-5 gene was not a member of the AP-1 family.

The Region between −70 and −59 is the major DNA-binding sequence in the 40-bp oligonucleotide. To further define the sequences that bound protein(s) within the 40-bp oligonucleotide, competitor oligonucleotides were derived by subdividing the 40-bp region into shorter overlapping sequences using the DNase I footprints as guides. One of these oligonucleotides contained sequences between −73 and −54, whereas the other included sequences between −91 and −74. Once again, in EMSAs with the 40-bp oligonucleotide as the probe, the upper complex shown in Fig. 3B (complex I in Fig. 4) was detected. A second specific complex (II), less abundant but largely inducible, was also detected (Fig. 4). The formation of both complexes was competed for by 100-fold excess of the 40-bp unlabeled oligonucleotide (lane 3) and the 20-bp oligonucleotide containing the sequence between −73 and −54 (lane 5). Neither of the oligonucleotides containing sequences between −91 and −74 or between −82 and −67 displayed any competition (lanes 4 and 9, respectively). This competition profile was completely compatible with the DNase I footprint shown in Fig. 2. The nature of proteins that generate the weaker footprint across −88 to −77 is unclear.

A close inspection of the sequence between −73 and −54 revealed the sequence AGATA, between nucleotides −70 and −65, which fit the consensus sequence WGATAR (W = A/T and R = A/G) for the GATA family of transcription factors. We noticed an overlapping TGATTG sequence on the complementary strand, which also was in close agreement with the GATA consensus sequence except for T at the +1 position. To investigate whether this region contributed to the formation of the DNA-protein complex, mutations were introduced in three different regions (m1, m2, and m3; see Fig. 4). m1 contained mutations in the distal GATA sequence, m2 in the proximal sequence, while m3 was mutated in both the sequences. Used

Fig. 2. Binding of EL-4 cell nuclear proteins to the IL-5 promoter between −188 and +1. DNase I protection experiments were performed with an IL-5 promoter fragment containing sequences between −188 and −24, labeled with 32P on the sense strand. Labeled DNA fragments were incubated without (lane O) or with nuclear extracts from EL-4 cells uninduced (lane 4) or with Bt2cAMP and PMA for 8 h (lane 1). The sequence from −88 to −41 is shown in detail with footprints identified by lines to the right of the figure.

family abolished most of the binding activity in complex II, but only weakly affected formation of complex I. The effect on complex III was similar to the effect of the anti-Jun antiserum (lane 11). An antibody against an unrelated transcription factor (p50 subunit of NF-κB) had no effect on any of the complexes (lane 12). Taken together, these results suggest that the major inducible CLE0 binding activity in complex II contains J unB, J unD, and a Fos family member. Complex I appears to be largely composed of J unB and J unD. However, since there was a small amount of residual DNA binding activity in both of these complexes that was not abolished by any of these antisera, we cannot rule out the possibility that there are less abundant complexes composed of related proteins. The identity of complex III (inducible or constitutive) is not known. Therefore, Fos and Jun proteins partially account for protein binding to the IL-5 CLE0 element in EL-4 cells stimulated with Bt2cAMP and PMA.

Bt2cAMP and PMA Induce Transcription Factor Binding to the Region Spanning Nucleotides −93 to −54—The region of the IL-5 promoter upstream of the CLE0 element contained critical functional activity and corresponded to a footprint within the region from −41 to −68 and a second weaker footprint between −88 and −77 (the former includes the AP-1-like sequence; Figs. 1C and 2). We used a radiolabeled 40-bp oligonucleotide containing the entire sequence between −93 and −54 (but excluding the CLE0 element) in the IL-5 promoter to investigate specific binding of proteins to this region in EMSAs (Fig. 3B). Two DNA-protein complexes were readily detected using this particular probe. The faster migrating complex, which could be detected using extracts from both unstimulated and stimulated cells, was clearly nonspecific in nature (Fig. 3B). However, the complex displaying slower electrophoretic mobility was less readily detectable when nuclear extracts were prepared from unstimulated cells, but its formation was significantly induced following stimulation of the cells for 8 h with Bt2cAMP and PMA (Fig. 3B, lane 1). A third complex (see Figs. 4 and 5), which was also largely inducible, was detected with excess nuclear extract. The complex shown in Fig. 3B represented a sequence-specific DNA binding activity because it was efficiently competed for by excess of the same unlabeled competitor oligonucleotide (Fig. 3B, lane 3) but not by oligonucleotides corresponding to the IL-5 CLE0 or the IL-5 NF-AT/CLE1 sites (lanes 4 and 7, respectively) or by the nonspecific competitors containing consensus AP-1 and NF-κB sites (lanes 5 and 6, respectively). Antibodies against the Jun and Fos family members also did not interfere with binding, further demonstrating that the transcription factor(s) binding to this region in the IL-5 gene was not a member of the AP-1 family.

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at a 100-fold molar excess, m2 appeared to compete better than the other two. When we repeated the experiment with 100- and 200-fold excess of the same oligonucleotides, the rank order of the ability to compete was m2 > m1 > m3 (Fig. 5). We also used consensus binding sequences for three additional transcription factor family proteins, C/EBP, Oct, and CREB/ATF, none of which, even at 200-fold excess, competed for binding to the probe (Fig. 5).

We next used oligonucleotides containing wild-type or mutant GATA sequence to investigate whether complexes I or II contained GATA proteins. As illustrated in Fig. 6, while an oligonucleotide containing wild-type GATA sequence displayed impressive competition, mutations within the GATA sequence abolished this competition. Since, among the GATA factors, GATA-3 has been reported to be predominantly expressed by T lymphocytes (37) and some expression also in the developing central nervous system, we used a GATA-3-specific antibody (which does not cross-react with either GATA-1 or 2), to further characterize the protein that bound to the AGATAA sequence in the IL-5 promoter. The anti-GATA-3 antibody but not the control NF-κB-specific antibody supershifted both the complexes (Fig. 6, lane 6). We also performed the reciprocal experiment using the oligonucleotide containing the wild-type GATA sequence as the probe. As is evident in lane 12, the IL-5 sequences between −73 and −54 efficiently competed for binding of GATA-3 to the consensus GATA sequence. The specific localization of the GATA-binding sequence to nucleotides between −70 and −59 in the IL-5 promoter also explained the complete loss of inducibility of the promoter when deleted to −66 (Fig. 1B).

While these studies were in progress, Yamagata et al. (43) reported the involvement of GATA-4 in the activation of the human IL-5 gene in the ATL-16T cell line. We, therefore, performed EMSA using a GATA-4-specific antibody that does not cross-react with any of the presently characterized GATA proteins (32). As illustrated in Fig. 7, while the anti-GATA-3 antibody again supershifted the specific DNA-protein complexes (lane 3), the anti-GATA-4 antiserum did not supershift or inhibit formation of either complex (lane 5). In the reciprocal situation as well, although anti-GATA-4 antiserum specifically inhibited complex formation by affinity-purified GST-GATA-4 (lane 8), anti-GATA-3 antibody had no effect on complex formation (lane 9). Taken together, these data suggest that GATA-3 (but not GATA-4) and Fos (JunB, JunD, the Fos family, and the p50 subunit of NF-κB), a radiolabeled oligonucleotide incorporating the region from −93 to −54 bp of the IL-5 promoter, exclusive of the potential NF-AT, CLE1, and CLE0 binding sites was used in EMSAs with nuclear extracts as described in A. An arrow identifies a band representing specific binding activity within this sequence, Competition with unlabeled "self," IL-5 CLE0, classical AP-1, NF-κB, and mutant CLE0 NF-AT/CLE1 oligonucleotides is shown. Antisera against the Jun and Fos families were utilized.

**DISCUSSION**

The results presented in this study demonstrate that sequences within the CLE0 element are critical but not sufficient for activation of the IL-5 promoter in response to cAMP agonists and phorbol esters. We have identified a second region between −70 and −59, which contains two overlapping GATA sites, disruption of which (as in the −66 deletion construct) abrogates activation of the promoter.

In agreement with a previous report by Lee et al., Bt2cAMP and PMA synergistically activated the IL-5 promoter (16). In a recent report, Lee et al. have implicated the NF-AT element in the IL-5 flanking region in induction of the gene by Bt2cAMP and PMA. In their studies, mutations in the NF-AT site led to an ~80% reduction in activation of the IL-5 promoter by these stimuli. Activation of NF-AT is coupled to increased levels of [Ca²⁺], (33, 38, 39). This requirement, however, can be bypassed in EL-4 cells due to constitutive increases in [Ca²⁺], (37). Our studies indicate that, although mutations in the NF-AT site in the context of the 545-bp promoter lead to ~50% reduction in activity of the promoter (which is approximately equivalent to an 80% reduction when compared to the 1.2-kilobase pair construct), the deletion construct with the end point just 3’ to this site (at −91) retains full transcriptional activity compared to the 545-bp construct, leading to a conclusion that the NF-AT site is not critical for induction of the promoter (Fig. 1B). In support of this, our DNaseI footprinting
studies did not reveal any binding of proteins across the NF-AT site in the IL-5 promoter, although distinct footprints were generated on the AP-1 and the GATA sites (Fig. 2). CsA blocks the nuclear translocation of NF-AT and has been shown to inhibit the expression of genes such as IL-2 and IL-4, which require NF-AT for induction of gene expression (33, 38, 39). Although Lee et al. (40) used CsA to demonstrate inhibition of binding of proteins to the NF-AT site in the IL-5 promoter, they did not investigate whether CsA indeed inhibits IL-5 gene expression in functional (transfection) experiments. Recently, Lacour et al. (41) reported inhibition of NF-AT induction by cAMP. Data previously reported by the Arai group also demonstrated that the NF-AT site in the IL-2 promoter is a target for inhibition of IL-2 promoter activation by cAMP, presumably via inhibition of calcineurin activity (42). However, this is only possible if distinct NF-AT species simultaneously control inhibition of the IL-2 gene and activation of the IL-5 gene by cAMP in the same cells (EL-4), as the authors also suggest (40).

Our studies establish a critical role for the GATA sequence in activation of the IL-5 promoter in EL-4 cells and also directly demonstrate binding of GATA-3 to the IL-5 promoter. In the studies reported by Lee et al. (40), deletion of the promoter to -62 and mutation of the GATA element led to complete abrogation of activation of the promoter by Bt2cAMP and PMA. We have established that the activation of the murine IL-5 gene in EL-4 cells involves binding of GATA-3, but not GATA-4, in contrast to the involvement of GATA-4 in the activation of the IL-5 gene in the ATL-16T cell line (43).

**Fig. 4.** The 20-bp oligonucleotide spanning nucleotides -73 to -54 (73/54) competes for binding of nuclear proteins to the longer 40-bp oligonucleotide. The probe was the same as in Fig. 3B. A 100-fold molar excess of the following unlabeled oligonucleotides were used as competitors: self (lane 3), two shorter oligonucleotides derived from this 40-bp oligonucleotide containing the region between -91 and -74 and between -73 and -54 (lanes 4 and 5, respectively), an overlapping oligonucleotide containing the sequence between -82 and -67 (lane 9), and three mutant oligonucleotides m1, m2, and m3 (lanes 6-8; the specific mutations are indicated by lowercase letters). All other conditions were as described in the legend to Fig. 3.

**Fig. 5.** Binding of nuclear proteins to the 20-bp 73/54 oligonucleotide. The competitor oligonucleotides m1, m2, and m3 were used at two concentrations, while the oligonucleotides containing the consensus sequences for the binding of C/EBP, Oct, and CREB/ATF family proteins were used at a 200-fold molar excess. All other conditions were as described in the legend to Fig. 3.

**Fig. 6.** Binding of GATA-3 to the 20-bp 73/54 oligonucleotide. The competitor oligonucleotides were used at a 100-fold molar excess. All other conditions were as described in the legend to Fig. 3.
GATA-3 and CLE0 in cAMP Activation of the IL-5 Promoter

**Fig. 7. GATA-3 and not GATA-4 is the IL-5 DNA-binding protein in EL-4 nuclear extracts.** 1 µl each of preimmune or immune serum was used in lanes 4 or 7 and lanes 5 or 8, respectively, while 0.2 µg of affinity-purified GST-GATA-4 protein was used in lanes 6–9. All other conditions were as described in the legend to Fig. 3.

GATA-3 and GATA-4 are members of the GATA family of transcription factors that bind to the consensus sequence -5WGATAR-3 via a highly conserved C4 zinc finger domain (44–46). Four GATA members, 1 through 4, have been described in vertebrates. The transcription factor GATA-3 is expressed most abundantly in T-lymphocytes and the developing central nervous system (46–50). In contrast, GATA-4 is predominantly expressed in the heart, gut epithelium, and reproductive organs (51–54). GATA-3 has been shown to play a crucial role in the transcriptional regulation of T cell receptor-related genes (46–49). DNA-binding studies with bacterially expressed GATA proteins and oligonucleotides containing randomized GATA sequences indicate that GATA sequences containing G at the +2 position, such as the one found in the IL-5 promoter between −70 and −65, may have a relatively lower binding affinity for the GATA-binding protein than sequences containing A at the +2 position (45). However, the data of Ko and Engel (45) indicate that the lower binding affinity of a site can be compensated for if this site overlaps another GATA site. Indeed, as shown in Fig. 1, the IL-5 gene does have overlapping GATA sites, one between −70 and −65 (which fits the consensus sequence) and another located between −65 and −59. Although the latter site has an intact GAT core but has a T instead of an A at the +1 position, it appears that this substitution can still be selected by GATA-3, particularly within overlapping GATA sites (45). Overlapping GATA sites have been also previously identified in many erythroid expressed genes such as the chicken a-globin promoter (55). Overlapping and or multiple GATA sites appear to confer increased GATA binding activity and may play a key role in promoting full transcriptional activity (55–57).

It remains undetermined from our data if both or one of the two potential GATA-3 sites are critical for protein binding. The finding that the m3 mutation, which disrupted both GATA sites, showed the least competition in our gel shifts suggests but does not prove that both sites may be critical for optimal binding. Both the IL-5 GATA sequence-containing probe and the oligonucleotide containing the GATA consensus sequence formed two complexes I and II with EL-4-nuclear extracts. It is possible that complexes I and II represent monomeric and dimeric forms (the latter induced by stimulation of cells) of the same protein. The requirement of the CLE0 element for activation of the promoter suggests that the GATA region alone is not sufficient for IL-5 promoter activation. In a similar fashion, in the T cell receptor-β gene, GATA-3 needs to interact with proteins bound to nearby sites for enhancer function (58).

Among the cytokine genes, potential GATA binding sequences are present in the 5'-flanking regions of the IL-3, IL-4, GM-CSF, and IFN-γ genes (but not in the IL-2 gene). There is no previous evidence for functional importance of this site in any of the other genes. In the case of the IFN-γ gene, deletion into the GATA sequence had little effect on activity of the promoter (59). To the best of our knowledge, this is the first report of a functional role for GATA-3 in the transcriptional regulation of a T cell cytokine gene. It will be interesting to determine in future studies with T cell clones and primary T lymphocytes whether human T cells utilize GATA-3 for inducible expression of the IL-5 promoter.

Naora et al. (60) have suggested that the TCTATT element, which overlaps with the AP-1-like element within the CLE0 element in the IL-5 promoter, is important for induction of IL-5 gene expression in response to mitogens and PMA. The TCA sequence in the TCTATT element overlaps with the AP-1 site, while the TTTT sequence overlaps with the Elf-1-binding site within the CLE0 element. In EMSAs reported by Naora et al. (60), mutation of the TCTATT element to cgAaTT (which also mutated the AP-1 element) abolished protein binding. However, the authors did not use antibodies against the AP-1 proteins to determine whether this complex contained AP-1 family proteins (60). It is possible that the TTTT, immediately adjacent to the AP-1 sequence, has a permissive role only (61, 62). Wang et al. (25) observed that the inducible transcription factors that bound to the GM-CSF CLE0 element contain J unB and c-Fos. Our gel shift assays show that specific antiserum to the AP-1 family members J unB and J unD and an antiserum that recognizes members of the Fos family significantly inhibit formation of complexes I and II with the IL-5 CLE0 element. However, since there was a small amount of residual DNA binding activity in both of these complexes that was not abolished by any of these antiseras, we cannot rule out the possibility that there are less abundant related proteins present in both the complexes. Complex III may comprise proteins similar to the constitutive proteins that Wang et al. had detected with the GM-CSF CLE0 probe (25).

Although the 5'-flanking region of the IL-5 gene bears some homology to the promoter of other cytokines such as GM-CSF and IL-4, the divergent regulation of these cytokines suggests that different mechanisms control their synthesis, particularly in response to elevations in intracellular cAMP levels (12, 13, 18, 63). Identification of a distinct combination of regulatory elements, i.e., GATA/CLE0 for cAMP activation of the IL-5 promoter, is therefore consistent with the differential effect of
cAMP on expression of the IL-5 gene. Ultimately, these findings may begin to explain how IL-5 gene expression is uniquely regulated by cAMP in T lymphocytes and how it becomes dysregulated in several common disease states.

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