

Of the GATA-Binding Proteins, Only GATA-4 Selectively Regulates the Human Interleukin-5 Gene Promoter in Interleukin-5-Producing Cells Which Express Multiple GATA-Binding Proteins

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Interleukin-5 (IL-5) is produced by T lymphocytes and known to support B-cell growth and eosinophilic differentiation of the progenitor cells. Using ATL-16T cells which express IL-5 mRNA, we have identified a region within the human IL-5 gene promoter that regulates IL-5 gene transcription. This *cis*-acting sequence contains the core binding motif, (A/T)GATA(A/G), for GATA-binding family proteins and thus suggests the involvement of this family members. In this report, we describe the cloning of human GATA-4 (hGATA-4) and show that hGATA-4 selectively interacts with the –70 GATA site within the IL-5 proximal promoter region. By promoter deletion and mutation analyses, we established this region as a positive regulatory element. Co-transfection experiments revealed that both hGATA-4 and phorbol-12-myristate-13-acetate (PMA)-A23187 stimulation are necessary for IL-5 promoter activation. The requirement for another regulatory element called CLE0, which lies downstream of the –70 GATA site, was also demonstrated. ATL-16T cells express mRNAs of three GATA-binding proteins, hGATA-2, hGATA-3, and hGATA-4, and each of them has a potential to bind to the consensus (A/T)GATA(G/A) motif. However, using ATL-16T nuclear extract, we demonstrated that GATA-4 is the only GATA-binding protein that forms a specific DNA-protein complex with the –70 GATA site. An electrophoretic mobility shift assay with extracts of COS cells expressing GATA-binding proteins showed that GATA-4 has the highest binding affinity for the –70 GATA site among the three GATA-binding proteins. When the transactivation abilities were compared among the three, GATA-4 showed the highest activity. These results demonstrate the selective role of GATA-4 in the transcriptional regulation of the IL-5 gene in a circumstance where multiple members of the GATA-binding proteins are expressed.

Interleukin-5 (IL-5) is a growth factor produced by T lymphocytes, and it promotes proliferation and differentiation of B lymphocytes and differentiation of eosinophil progenitors (31, 35). The human IL-5 genomic locus has been cloned and found to be localized to chromosome 5q, forming a cluster with the genes of other growth factors such as IL-3, IL-4, IL-9, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (16, 36). The nucleotide sequence of its 5'-flanking region upstream of the transcription initiation site was also determined and characterized (36). There is a short motif called CLE0, between nucleotide positions –56 and –42 within the human IL-5 promoter region, which is highly conserved among the 5' upstream regions of several cytokine genes such as the IL-3, IL-4, GM-CSF, granulocyte colony-stimulating factor (G-CSF), and mouse IL-5 genes (20, 23, 24). This element is known to mediate response to the T-cell activation signal that is mimicked by treatment with phorbol-12-myristate-13-acetate (PMA) and calcium ionophore (A23187) (20, 23). Although the functional involvement of the CLE0 element in response to PMA-A23187 stimulation has been demonstrated to occur in

the GM-CSF promoter, a previous study showed that treatment with PMA-A23187 is not sufficient to activate the IL-5 promoter (20, 22). These facts prompted us to investigate the novel molecular mechanism of IL-5 gene regulation and the functional role of the IL-5 CLE0 element in the context of the human IL-5 proximal promoter.

To investigate this issue, we used ATL-16T cells, a T-cell line derived from a patient with adult T-cell leukemia (ATL) (28), because it constitutively expresses IL-5 mRNA. We transfected the cells with various IL-5 promoter-luciferase constructs. By promoter deletion analysis, the region from nucleotide –82 to –62, named region G1, has been revealed to carry a major promoter-enhancing activity (26a). In an electrophoretic mobility shift assay (EMSA), the nuclear extract prepared from ATL-16T cells produced a shifted band with labeled oligonucleotides of this region, suggesting that the DNA-binding protein which binds to this region carries a promoter-enhancing activity. Since region G1 contains the AGATAG consensus sequence for the GATA-binding family proteins, we hypothesized the involvement of the GATA-binding family proteins in IL-5 gene regulation. Six members of the GATA-binding family proteins have been identified so far and are designated GATA-1/Eryf1 (6, 39), GATA-2 (5, 17, 42), GATA-3 (7, 9, 12, 42), GATA-4 (1), GATA-5 (10, 15), and GATA-6 (15). Each of these factors is known to be expressed in restricted tissues, and their important roles have been demonstrated. GATA-1

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expression is found in hematopoietic tissues, specifically in erythroid cells, megakaryocytes, eosinophils, and bone marrow-derived mast cells, and in a nonhematopoietic tissue, the testes (6, 8, 19, 39, 45). More widely expressed GATA-2 is found in endothelial cells, erythroid progenitors, megakaryocytes, mast cells, monocytic cells, and fibroblasts (5, 17, 42). GATA-3 is expressed in T cells, mast cells, embryonic brains, and kidneys (7, 9, 12, 42). GATA-4 is detected in the heart, ovaries, testes, and certain endodermal derivatives (1). A study demonstrated that GATA-4 regulates cardiac muscle-specific expression of the α -myosin heavy-chain gene (25). Studies of recently cloned GATA-5 and GATA-6 suggested their role in cardiac and gastrointestinal development (10, 15).

To isolate and characterize the protein suggested to be involved in the region G1-dependent induction of the IL-5 gene promoter, we screened a bacteriophage λ expression cDNA library of ATL-16T cells. We found that three GATA binding proteins, GATA-2, GATA-3, and GATA-4, are expressed in ATL-16T cells. We showed that the IL-5 promoter preferentially interacts with and is transactivated by GATA-4. This is the first study demonstrating the functional selectivity of a single member of the GATA-binding family protein in specific gene regulation, although the difference in binding affinity for the recognition core sequences was previously suggested by in vitro binding assays (11). In addition to the functional involvement of GATA-4, we found a requirement for an IL-5 CLEO element whose role was not fully understood in earlier studies (22, 23). The possible mechanism of IL-5 gene regulation and the significance of the multiple members of the GATA-binding family proteins are discussed in this report.

MATERIALS AND METHODS

Cell lines. ATL-16T (28), Jurkat, PEER, T-ALL-1, CCRF-CEM, MOLT-3, SKW-3 (T-cell lymphoblastic leukemia), B-ALL-1 (B-cell lymphoblastic leukemia), Ramos, Raji (Burkitt lymphoma), K812, HEL (erythroleukemia), U-937 (histiocytic lymphoma), HLCL-1 (peripheral lymphocyte), K562 (chronic myelogenous leukemia), HL-60 (promyelocytic leukemia), and Josk-K (monocytic leukemia) cells were maintained in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C in 5% CO₂. COS cells and HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and penicillin (100 U/ml) at 37°C in 5% CO₂.

Plasmids and oligonucleotides. pUC00Luc was constructed by replacing the chloramphenicol acetyltransferase (CAT) gene of pUC00CAT (13) with the firefly luciferase (Luc) gene. A region of the human IL-5 promoter gene (36) from -400 to +20 relative to the transcription initiation site was fused upstream of the firefly luciferase gene of pUC00Luc to generate pUC-hIL5Luc. *SalI* (GTTCGAC)-replacing mutations were introduced into specific sites in pUC-hIL5Luc by site-directed mutagenesis (Muta-Gene; Bio-Rad). As a result, the *SalI* mutation constructs pUC-hIL5Luc (m-110), (m-76), (m-70), (m-45), and (m-35) were generated. A series of promoter deletion constructs was generated by removing the 5' upstream region of the introduced *SalI* site, resulting in the deletion constructs pUC-hIL5Luc (Δ -110), (Δ -76), (Δ -70), (Δ -45), and (Δ -35). Eukaryotic expression vector pSSR α -hGATA-4 was prepared by cloning the filled 1.7-kb *EcoRI-SalI* fragment of hGATA-4 cDNA into the filled *EcoRI* site of pSSR α (38).

The herpes simplex virus thymidine kinase (*tk*) promoter (18) fused to the luciferase gene (*tk*-Luc) was constructed as described previously (37). Six tandem copies of region G1 [(GGCATTCTCTATCTGATTGTT)₆] were fused upstream of the *tk* promoter region of *tk*-Luc to generate p(G1)₆-*tk*-Luc. Similarly, six tandem copies of region G1 with a mutated GATA site [(GGCATTCTGTCGACGATTGTT)₆] were used to generate p(mG1)₆-*tk*-Luc. Four tandem copies of the T-cell receptor α (TCR α)-GATA sequence (see below) were used to generate p(TCR α -GATA)₄-*tk*-Luc.

The sense strands of oligonucleotides used for EMSA are shown in Table 1.

Southwestern (DNA-protein) screening of λ gt11 expression library and production of recombinant protein. The λ gt11 expression library of ATL16 was prepared by using the You-Prime cDNA synthesis kit (Pharmacia). The Southwestern screening was done as described previously (32). As for a screening probe, the sense and antisense nucleotide sequences at position -82 to -62 (GGCATTCTCTATCTGATTGTT) of the IL-5 promoter were synthesized, phosphorylated on their 5' ends, and ligated to generate the repeated polymers by tandem ligation. Six repeats of polymers [(GGCATTCTCTATCTGATTGTT)₆]

TABLE 1. Sense strands of oligonucleotides used for EMSA^a

Oligonucleotide	Sense strand (-82 to -62)
G1-wild.....	5'-GGCATTCTCTATCTGATTGTT-3'
G1-mut1.....	5'-GGCATTCTGTCCGACGATTGTT-3'
G1-mut2.....	5'-GTCCGACCTCTATCTGATTGTT-3'
G1-mut3.....	5'-GGCAGTCTCTATCTGATTGTT-3'
G1-mut4.....	5'-GGCATTCTCTA <u>GT</u> CTGATTGTT-3'
G1-mut5.....	5'-GGCATTCTCTATCTGAGTGT-3'
TCR α -GATA.....	5'-GTTAGAGATAGCATCGCCCCA-3'

^a Mutated nucleotides are underlined. The GATA sequence of the human TCR α enhancer (T α 3) is also listed (7).

TT)₆] were excised from a low-melting-point agarose gel (Sigma) and subcloned into the *EcoRV* site of Bluescript (Stratagene). The fragment was excised from the plasmid DNA by *PstI-HindIII* digestion, labeled by Klenow reaction with [α -³²P]dCTP (Amersham), and used as a probe. The extracts derived from the resulting positive clones were prepared as described previously (21). These extracts were used for EMSA to confirm the DNA binding activity of the encoded proteins. The cDNA fragments of positive clones were labeled and used as probes to further screen an oligo(dT)-primed cDNA library to obtain full-length clones.

Sequence analysis of human GATA-4 cDNA. The longest clone was analyzed for determination of the nucleotide sequence of human GATA-4 cDNA. The constructs of nested deletions on both strands were generated, and nucleotide sequencing was performed by using Sequenase (U.S. Biochemical).

Antisera. Antisera for each GATA-binding protein were raised against glutathione S-transferase (GST) fusion proteins. For each GST fusion construct, a part of the portion outside the DNA binding domain of the GATA-binding protein was subcloned into GST fusion expression plasmids. The GST-hGATA2 plasmid was constructed by in-frame cloning of the 5'-*NcoI* (nucleotide 192)-*SacI* (nucleotide 639)-3' fragment into pGEX-2T (Pharmacia). The GST-hGATA3 plasmid was constructed by in-frame cloning of the 5'-*NcoI* (nucleotide 198)-*BamHI* (nucleotide 473)-3' fragment into pGEX-2T. The GST-hGATA4 plasmid was constructed by in-frame cloning of the 5'-*BamHI* (nucleotide 1234)-*SalI* (nucleotide 1760)-3' fragment into pGEX-3X. Expression and partial purification of each of the fusion proteins were carried out according to published procedures (2, 33). Partially purified fusion proteins were used to prepare antisera via subcutaneous injection of rabbits.

Preparation of cell extracts and EMSA. COS cells were transfected by the DEAE-dextran method (30). Extracts from transfected COS cells were prepared as described previously (14). Preparation of nuclear extracts from ATL-16T cells and EMSA were done as described previously (27). Briefly, 1 μ g of COS cell extract or ATL-16T cell nuclear extract was incubated with poly(dI-dC) (Pharmacia) and labeled G1 oligonucleotides in EMSA binding buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 12.5% glycerol). The labeled G1 oligonucleotides were prepared by filling in the 21-bp double-stranded oligonucleotides encoding the -82 to -62 region of the human IL-5 promoter with Klenow fragment and [α -³²P]dCTP (Amersham). TCR α -GATA oligonucleotides were labeled in the same way. For competition experiments, each unlabeled oligonucleotide harboring mutations was added to the reaction mixture prior to the addition of the labeled oligonucleotides. For the antiserum supershift experiment, each antiserum was added to the extracts and incubated for 15 min on ice prior to the addition of the labeled oligonucleotides.

RNA isolation and Northern analysis. RNA was prepared from tumor cell lines by the acid guanidinium thiocyanate-phenol-chloroform method (4). Poly(A)⁺ RNA was selected by oligo(dT)-cellulose (Pharmacia). Northern (RNA) blotting was performed as described by Maniatis et al. (30). Five micrograms or 1.5 μ g of poly(A)⁺ RNA was electrophoresed through a 1.0% agarose gel containing 2.0 M formaldehyde, 40 mM MOPS (morpholinepropanesulfonic acid), 5 mM sodium acetate, and 0.5 mM EDTA (pH 7.0) and subsequently transferred onto Hybond N nylon membranes (Amersham) in 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Blots were hybridized with a ³²P-labeled DNA fragment from human GATA-1 (hGATA-1) (44), a 400-bp *PstI-HindIII* fragment from the N terminus coding region; hGATA-2 (5), a 770-bp *PvuII-EcoRI* fragment spanning from the C terminus coding region to the 3' untranslated region; hGATA-3 (7), an 820-bp *SalI-EcoRI* fragment spanning from the C terminus coding region to the 3' untranslated region; and hGATA-4, a 950-bp fragment spanning from the 5' untranslated region to the *HincII* site at nucleotide position 947. Prehybridization and hybridization were performed at 42°C in a solution containing 50% formamide, 5 \times SSC, 5 \times Denhardt's solution, 20 μ g of salmon sperm DNA per ml, and 0.5% sodium dodecyl sulfate (SDS). Filters were washed three times for 15 min with 0.1 \times SSC-0.1% SDS at 55°C. Autoradiography was performed on Kodak X-Omat AR film with an intensification screen at -70°C for 24 h.

DNA transfection and luciferase assay. Transfection and stimulation of Jurkat cells were done as described previously (27). Briefly, 10⁷ cells in the logarithmic

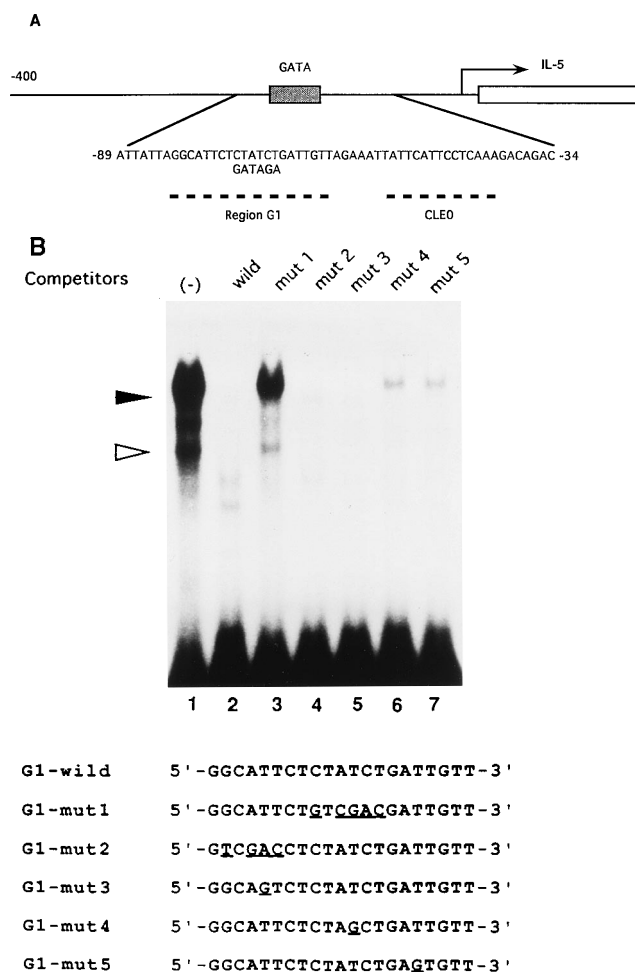


FIG. 1. (A) Structure of the human IL-5 gene promoter. G1 indicates the position of the G1 oligonucleotide used for screening the λ gt11 expression library and EMSA. The -70 GATA and -56 to -42 CLE0 elements are also indicated. (B) Identification of the protein that specifically interacts with the G1 oligonucleotide. ATL-16T nuclear extract was incubated with the labeled G1 oligonucleotides with or without cold competitors indicated above each lane. The closed arrow indicates the slowly migrating complex, and the open arrow indicates the fast migrating complex. The nucleotide sequences of the competitors are listed below.

growing phase were incubated for 25 min at room temperature with 500 μ g of DEAE-dextran (Pharmacia) per ml, 10 μ g of reporter plasmid DNA, and 5 μ g of pSSR α -hGATA4. Subsequently, the cells were incubated for 1 h at 37°C in 5% CO₂ in culture medium containing 100 μ M chloroquine. The transfected cells were incubated in culture medium for 36 h and stimulated with 20 ng of PMA (Sigma) per ml and 500 nM A23187 (Calbiochem) for another 12 h. Cells were harvested for the luciferase assay according to the manufacturer's instructions (Luciferase Assay System; Promega). Twenty microliters of cell extracts was used for each assay.

NIH 3T3 cells were transfected by the calcium phosphate method (30). Five micrograms of the reporter construct and 3 μ g of the expression plasmid were cotransfected. Cells were harvested 48 h posttransfection and subjected to the luciferase assay. Each signal value (in relative light units) was adjusted for each protein concentration.

RESULTS

Identification of the protein that binds to region G1 in ATL-16T nuclear extract. Region G1 (Fig. 1A) was identified during an attempt to delineate regions within the human IL-5 proximal promoter that interacts with a factor(s) present in the nuclear extracts prepared from the IL-5-expressing cell line ATL-16T (28). It was also suggested, by promoter deletion

analysis with ATL-16T cells, that the factor binding to region G1 is involved in IL-5 promoter regulation (26a). An EMSA with the G1 oligonucleotides showed a fast-migrating complex (Fig. 1B, closed arrow) and a slowly migrating complex (Fig. 1B, open arrow) in the nuclear extract of ATL-16T cells. To identify the nucleotide sequence involved in these DNA-protein interactions, 60-fold excesses of cold competitors with mutations at different sites of the G1 oligonucleotides were coinubated and subjected to EMSAs (Fig. 1B). A competitor with five base mutations within the consensus binding sequence for the GATA-binding family proteins (mut1) did not inhibit the slowly migrating upper complex (lane 3). A competitor harboring a single base mutation within the consensus binding site (mut4) could not inhibit the complex completely (lane 6) as did the wild type, mut2, and mut3 (lane 2, lane 4, and lane 5, respectively). These results suggested that DNA-binding proteins that have high affinity for the AGATAG sequence, especially the GATA-binding family proteins, could be involved in this DNA-protein complex. The partial inhibition observed with the competitor harboring a mutation at the 3' end flanking the GATA site (mut5) suggested that the flanking nucleotide also affects the interaction between the core motif and its binding protein (lane 7). The fast-migrating lower band seems to be a degraded product of the upper complex since the repeated freeze-and-thaw treatments of the nuclear extracts decreased the upper complex and increased the lower one.

Southwestern screening reveals that the three GATA-binding proteins (GATA-2, GATA-3, and GATA-4) are expressed in ATL-16T cells. There are six members (GATA-1 to GATA-6) of the GATA-binding family proteins identified so far, and each of them possibly gives rise to the G1-protein complex in EMSAs. In addition, there are other transcription factors that recognize the GATA motif as their recognition consensus sequences but are not classified as members of the GATA-binding family proteins, such as Evi-1 (29). Therefore, it was difficult to predict the G1-binding protein just from its binding nucleotide sequence. To identify such proteins and isolate their cDNAs, we employed the Southwestern screening method. A probe that has six tandem repeats of the G1 sequence [(GGCATTCTCTATCTGATTGTT)₆] was used to screen 250,000 independent plaques of a λ gt11 cDNA expression library of ATL-16T cells. As a result, five positive clones were isolated. To confirm that the obtained clones encode the proteins capable of binding to a monomeric G1 probe, the extracts from these lysogens were prepared and subjected to a gel retardation assay using the monomeric α -³²P-labeled G1 oligonucleotides. Four extracts gave rise to shifted bands, but one did not (data not shown). We speculate that the latter one recognized an adjacent sequence originating as a result of the tandem ligation. Sequencing and homology searches of the four positive clones revealed two clones encoding partial fragments of hGATA-2 (5, 17) and one clone encoding a fragment of hGATA-3 (7, 9, 12). The last one contained a novel sequence not found in databases but homologous with the sequence of mouse GATA-4 (1), suggesting that it is a human counterpart. This was confirmed by the complete nucleotide sequence analysis of the whole coding region of hGATA-4 (see Fig. 3B). The identification of the GATA-binding family proteins in Southwestern screening was consistent with the results of binding competition experiments in EMSAs (Fig. 1B). To confirm the expression of these three GATA-binding family proteins in ATL-16T cells, we investigated the mRNA expression of the four members of the GATA-binding family proteins by Northern analysis. ATL-16T cells did not express GATA-1 but did express GATA-2, GATA-3, and GATA-4 in roughly similar amounts (Fig. 2). This result was consistent with that of

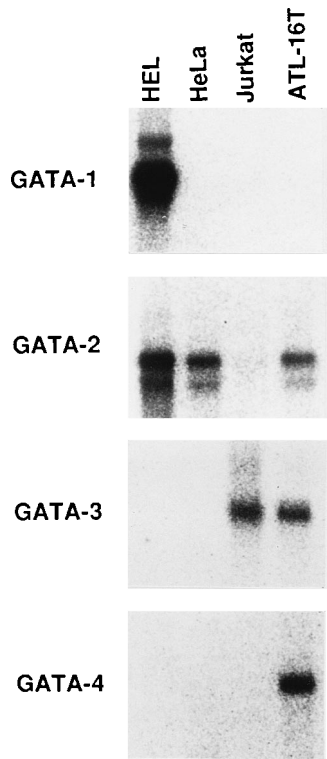


FIG. 2. Northern analysis of the genes encoding human GATA-binding proteins expressed in ATL-16T cells. Each of 1.5- μ g poly(A)⁺ RNAs derived from HEL, HeLa, Jurkat, and ATL-16T cells was hybridized to ³²P-labeled cDNA probes encoding hGATA-1, hGATA-2, hGATA-3, and hGATA-4. RNAs from HEL, HeLa, and Jurkat cells served as positive controls for GATA-1, GATA-2, and GATA-3, respectively. Every cDNA probe does not contain the sequence encoding highly conserved DNA-binding domains in order to prevent cross-hybridization between the different genes for the GATA-binding proteins and the cDNA probes.

the Southwestern screening, which identified GATA-2, GATA-3, and GATA-4 but not GATA-1.

Nucleotide sequence of hGATA-4. To further investigate the functional role of hGATA-4, we screened an oligo(dT)-primed cDNA library of ATL-16T and isolated a full-length cDNA of hGATA-4. The nucleotide sequence of this cDNA clone contained an open reading frame encoding 439 amino acids, with a predicted molecular mass of 45.3 kDa (Fig. 3A). High homology with the previously cloned mouse GATA-4 demonstrated that this is a human homolog of mouse GATA-4. The ATG beginning at nucleotide position 302 is presumed to be a start codon, and this is consistent with the mouse counterpart. An in-frame stop codon is present 126 nucleotides upstream of the predicted start codon. Comparison of mouse and human GATA-4 amino acid sequences showed 82.5% identity, indicating that GATA-4 is highly conserved between these two species (Fig. 3B).

Expression pattern of GATA-4 in hematopoietic cell lines. The previous studies demonstrated that GATA-4 is expressed in the heart, ovaries, and testes and in certain endodermal derivatives (1). Its specific expression in cardiac muscle and important roles in cardiogenesis were also suggested (25). Although it has been indicated that GATA-1, GATA-2, and GATA-3 have important roles in hematopoiesis, the role of GATA-4 in hematopoiesis has not been studied yet. The detection of GATA-4 mRNA in ATL-16T cells prompted us to investigate its expression patterns in other hematopoietic cell

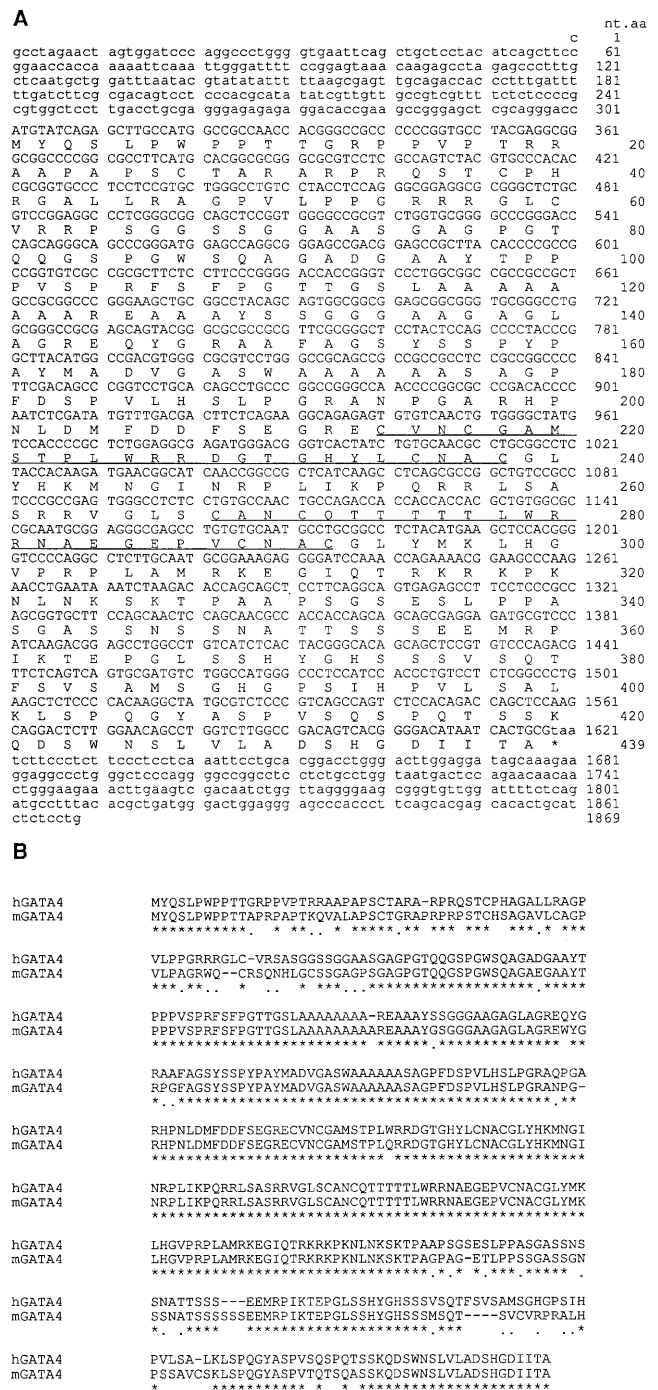


FIG. 3. (A) Nucleic acid and predicted amino acid (aa) sequences of human GATA-4. The nucleotide (nt) numbers from the 5' end of the cloned cDNA and the amino acid numbers from the first Met of the single open reading frame are shown on the left. (B) Comparison of human and mouse GATA-4 amino acid sequences. Comparison of the human and mouse GATA-4 sequences shows 82.5% identity, which is marked by asterisks. Dots indicate similar amino acids, and bars indicate skipped amino acids.

lines. Interestingly, it was shown that the 3.3-kb mRNA of human GATA-4 is expressed only in ATL-16T cells and not in other hematopoietic cell lines (Fig. 4). This result is consistent with a previous study of mouse GATA-4 which showed no expression of GATA-4 in cell lines of hematopoietic origin (1).

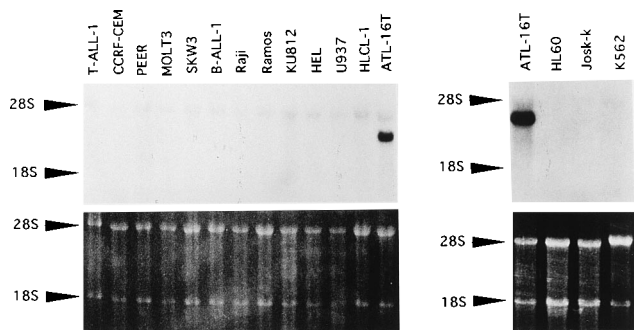


FIG. 4. Northern analysis of human GATA-4 expression in hematopoietic cell lines. For each panel, 5 μ g of poly(A)⁺ RNA from the cells indicated at the top was hybridized with the GATA-4 cDNA probe as described in Materials and Methods. RNA from ATL-16T cells was blotted on both filters to serve as a positive control. Triangles indicate the positions of 28S and 18S rRNA. Ethidium bromide staining of the electrophoresed gels is shown below each blot.

Of the three GATA-binding proteins, GATA-4 is the only one that forms specific a DNA-protein complex with the G1 sequence. To determine which of the three GATA-binding family proteins (GATA-2, GATA-3, and GATA-4) of ATL-16T nuclear extracts forms shifted bands with the G1 oligonucleotides in EMSAs, we raised antisera against hGATA-2, hGATA-3, and hGATA-4, respectively. Each antiserum was raised against a region outside the conserved DNA binding domain of GATA-binding family proteins to discriminate each member of the GATA-binding family proteins. The ability of each antiserum to supershift the corresponding specific DNA-

protein complex was confirmed by using recombinant GATA-2, GATA-3, or GATA-4 protein expressed in COS cells (Fig. 5A). Antiserum raised against hGATA-2 or hGATA-3 clearly supershifted the DNA-hGATA-2 or DNA-hGATA-3 complex (Fig. 5A, lane 4 and lane 7). Antiserum raised against hGATA-4 partially inhibited and partially supershifted the G1-GATA-4 complex (lane 10). An increased amount of the antiserum completely inhibited the G1-GATA-4 complex (lane 11 and lane 12). No supershifted band of the G1-protein complex was observed with each preimmune serum. Next, each of the established antisera was incubated with nuclear extract from ATL-16T cells and labeled G1 oligonucleotides and subjected to an EMSA. Antiserum raised against hGATA-2 or hGATA-3 did not supershift the upper complex (Fig. 5B, lane 3 and lane 5). However, anti-hGATA-4 antiserum supershifted and inhibited the upper band (lane 7), as was observed with recombinant hGATA-4 expressed in COS cells (Fig. 5A, lane 10). An increased amount of anti-hGATA-4 antiserum completely inhibited the upper specific band (lane 8 and lane 9). These results indicate that GATA-4 forms specific a G1-protein complex in EMSAs using ATL-16T nuclear extract. It is interesting that this complex does not contain hGATA-2 or hGATA-3 even though they were expressed in ATL-16T cells.

Recombinant GATA-4 exhibits an inhibition pattern in EMSAs similar to that observed with ATL-16T nuclear extract. To investigate whether the complex formed by GATA-4 and G1 oligonucleotides exhibits the same inhibition pattern as that observed in Fig. 1B, we performed EMSAs with the recombinant GATA-4 and the various cold competitors. As shown in Fig. 6, the competitors harboring mutations at the GATA site (mut1 and mut4) did not inhibit the complex (Fig.

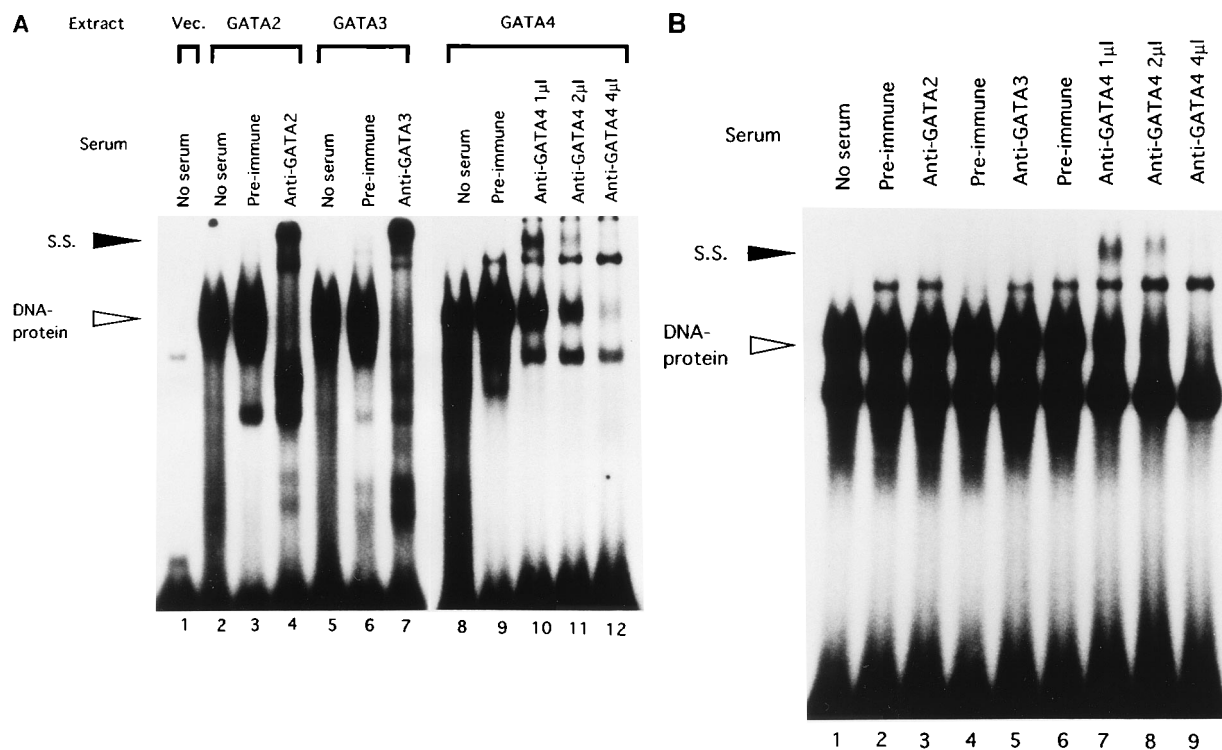


FIG. 5. EMSA with antisera raised against each GATA-binding protein. The extracts and antisera employed are indicated above the panels. Each extract was preincubated for 15 min on ice with the indicated antiserum and subjected to EMSA. (A) Labeled oligonucleotides for TCR α were used for lane 1 to 7; labeled G1 oligonucleotides were used for lanes 8 to 12 as a probe. Each DNA-GATA-binding protein complex is indicated by an open arrow. The supershifted (S.S.) complex is indicated by a closed arrow. (B) Labeled G1 oligonucleotides were used for all lanes. The DNA-protein complex is indicated by an open arrow. The supershifted complex is indicated by a closed arrow.

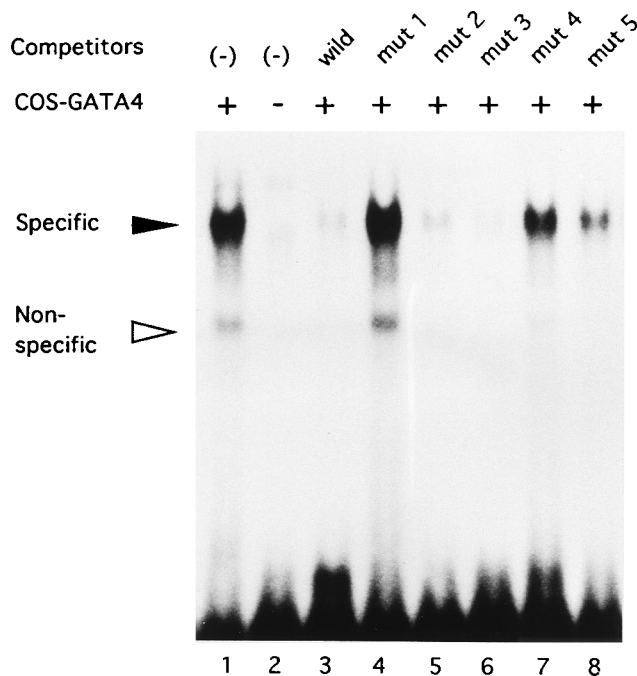


FIG. 6. EMSA of the labeled G1 oligonucleotides and extract of the COS cells expressing GATA-4. For each lane, 1 μ g of extract of the COS cells transfected with pSSR α -hGATA4 was incubated with radiolabeled G1 oligonucleotides in the presence or absence of a 60-fold molar excess of cold competitors. Lanes 1 and 2, without competitors; lane 3, G1-wild; lane 4, mut1; lane 5, mut2; lane 6, mut3; lane 7, mut4; lane 8, mut5. A closed arrow indicates the G1-GATA-4 complex.

6, lane 4 and lane 7) while those harboring mutations outside the GATA site inhibited the binding (lane 5 and lane 6). The competitor mut5, which has a mutation in the 3' flanking sequence of the GATA site, could not completely inhibit the complex (lane 8), indicating that the 3' flanking sequence of the GATA site is also involved in the specific DNA-protein interaction. This inhibition pattern is similar to that observed in the inhibition experiment with ATL-16T nuclear extract (Fig. 1B). In addition, the G1-GATA-4 complex formed with the COS cell extract migrated to the same position as the upper complex formed with ATL-16T nuclear extract in EMSAs (data not shown). These data suggest that the upper complex observed in EMSAs with G1 oligonucleotides and ATL-16T nuclear extract consists of hGATA-4.

The G1-GATA site is a positive regulatory element. To determine whether GATA-4 enhances transcription through interacting with the G1-GATA site, we cotransfected Jurkat cells, in which GATA-4 is not expressed, with pSSR α -hGATA4 and the reporter constructs containing six tandem copies of the region G1 fused to the *tk* promoter, designated (G1)₆-*tk*. Luciferase activity driven by this chimeric (G1)₆-*tk* promoter increased by 13-fold, while the chimeric promoter carrying mutations within the GATA sites, designated (mG1)₆-*tk*, exhibited a twofold increase in the presence of GATA-4 (Fig. 7). The latter effect seems to be nonspecific since pSSR α -hGATA4 stimulated the expression of the herpes simplex virus thymidine kinase promoter (*tk*-Luc) roughly twofold. These results indicate that GATA-4 can act through the G1-GATA site to enhance transcription from a heterologous promoter.

GATA-4 transactivates the IL-5 promoter through interaction with the -70 GATA site. Next, we addressed whether GATA-4 is capable of transactivating the native IL-5 promoter

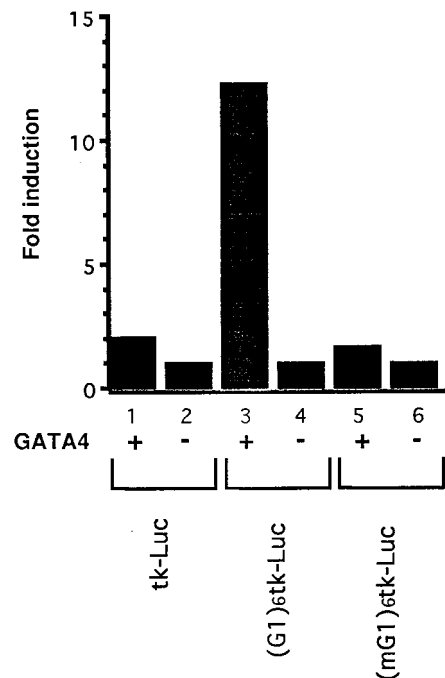


FIG. 7. The G1-GATA element is a positive regulatory element in a heterologous promoter. Jurkat cells were cotransfected with pSSR α -hGATA4 and heterologous promoter-luciferase constructs which are described in Materials and Methods. The transfected cells were not treated with PMA-A23187. The fold induction compared to that in the absence of GATA-4 is indicated for each reporter construct.

in Jurkat cells which do not express IL-5 or GATA-4. Jurkat cells were cotransfected with pSSR α -hGATA-4 and the -400 IL-5 promoter fused to the luciferase reporter (pUC-hIL5Luc) (Fig. 8A). Contrary to our expectation, however, an enhanced luciferase activity was not observed in the cotransfection experiment (Fig. 8A, lane 3). Taken together with the results of the experiments using the heterologous promoter (Fig. 7), these results led us to speculate that although GATA-4 has the potential to transactivate the IL-5 promoter, an additional factor may be required for the full transactivation of the native IL-5 promoter. T-cell activation can be mimicked by treatment with PMA and A23187. The studies of the GM-CSF promoter demonstrated that this signal is mediated by the CLE0 element of the promoter (20), and the highly conserved motif also lies within the IL-5 promoter region (Fig. 1A). Therefore, we stimulated the pSSR α -hGATA-4- and pUC-hIL5Luc-cotransfected Jurkat cells with PMA-A23187. In the presence of PMA-A23187 stimulation, GATA-4 enhanced the promoter activity 8.5-fold (Fig. 8A, compare lane 1 and lane 2) whereas PMA-A23187 stimulation alone did not increase the IL-5 promoter activity (lane 4). This result indicates that the IL-5 promoter requires GATA-4 for its activation but that GATA-4 alone is not sufficient to enhance the promoter activity.

To examine whether the observed transactivation of the IL-5 promoter was mediated through the -70 (G1) GATA site, a deletion analysis of the IL-5 promoter was carried out. A series of reporter constructs was investigated in the presence of GATA-4 and PMA-A23187 stimulation. Deletion upstream to nucleotide -76 relative to the transcription initiation site did not significantly affect induction by PMA-A23187 and GATA-4 in Jurkat cells (Fig. 8B, Δ -76). However, deletion upstream to -70, which removes the -70 GATA site, essen-

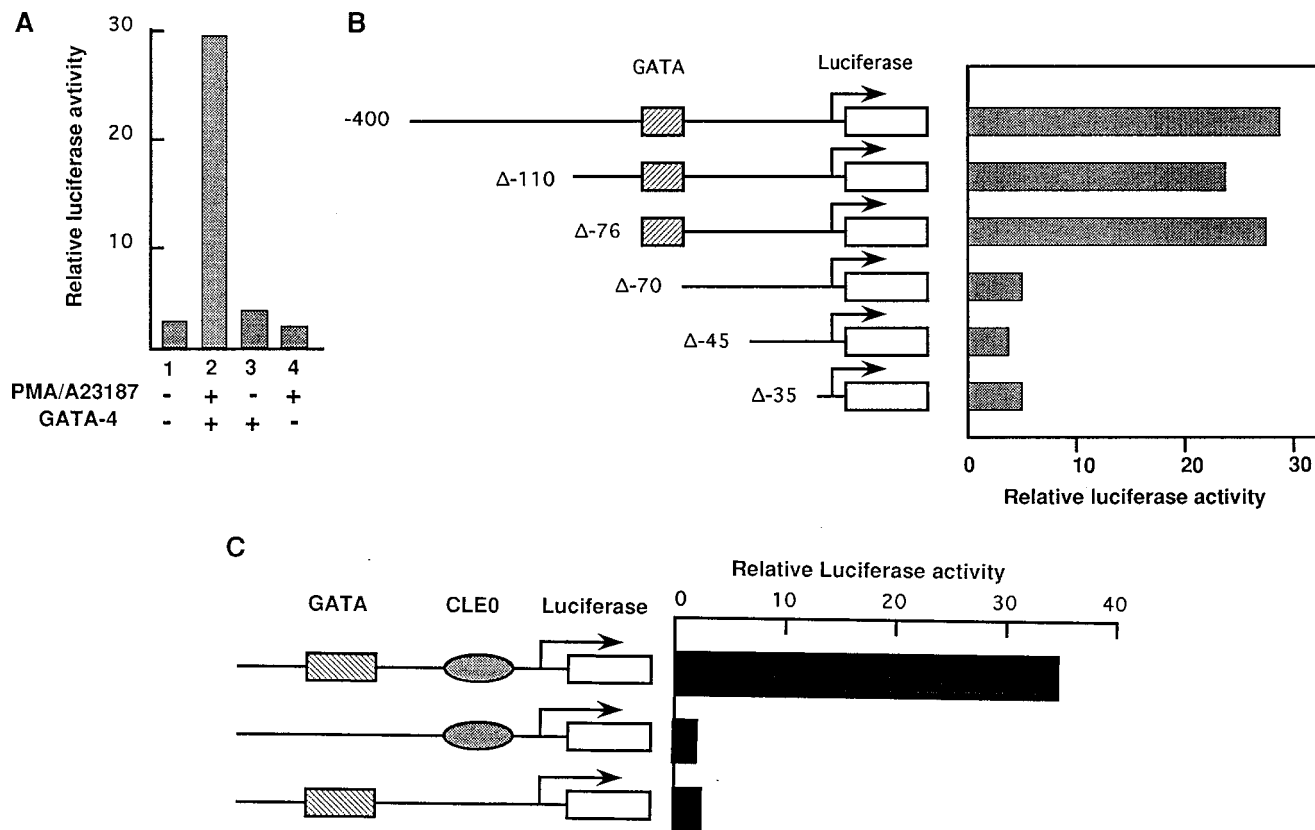


FIG. 8. Transactivation of the native human IL-5 promoter-luciferase construct by hGATA-4. Jurkat cells were cotransfected with pSSR α -hGATA4 and reporter plasmids as described in Materials and Methods. (A) Both GATA-4 and PMA-A23187 are required to activate the native IL-5 promoter (pUC-hIL5Luc). (B) Deletion analysis of the native IL-5 promoter in the presence of GATA-4 and PMA-A23187 treatment. (C) Mutation analysis of the -70 GATA element (m-70) and the CLE0 element (m-45) of the native IL-5 promoter in the presence of PMA-A23187 treatment. For each transfection, the experiment was performed three times and the results were essentially reproducible.

tially abolished the PMA-A23187- and GATA-4-induced transactivation of the IL-5 promoter (Fig. 8B, compare Δ -76 and Δ -70). These results indicate that the -70 GATA site mediates the transactivation of the IL-5 promoter by GATA-4.

Both the -70 GATA and -56 to -42 CLE0 elements are necessary for IL-5 promoter activation. To further confirm that the -70 GATA site is essential for GATA-4-mediated transactivation and to clarify the functional role of the CLE0 element, constructs harboring mutations within the -70 GATA site or the CLE0 element were examined. As expected, a mutation introduced in the -70 GATA site abolished the PMA-A23187- and GATA-4-mediated transactivation (Fig. 8C). These results confirm that GATA-4 transactivates the IL-5 promoter through interaction with the -70 GATA site. Transactivation of the IL-5 promoter was also abolished when mutations were introduced within the CLE0 element (Fig. 8C). These results indicate that both the -70 GATA element and the CLE0 element are necessary for the transactivation of the IL-5 promoter.

In cotransfection experiments (Fig. 7 and Fig. 8), the effect of endogenous GATA-3 in Jurkat cells could be ignored since the transfection of pUC-hIL5Luc in the presence of PMA-A23187 stimulation gave almost the same luciferase activity as that in the absence of the stimulation (Fig. 8A, compare lane 1 and lane 4).

GATA-4 preferentially binds to and transactivates the IL-5 promoter. Although the G1 oligonucleotide contains the (A/T)GATA(A/G) consensus sequence for the GATA-binding

family of proteins, the results of the supershift experiments showed that GATA-2 and GATA-3 were not the components of the complex. This fact led us to speculate that GATA-2 and GATA-3 might have low affinities for the G1-GATA sequence compared with that of GATA-4. To examine this hypothesis, we performed an EMSA with identical amounts of recombinant GATA-2, GATA-3, GATA-4 proteins. As we expected, GATA-2 and GATA-3 did not form strong shifted complexes as GATA-4 did with the labeled G1 oligonucleotides (Fig. 9A). The result was reversed when similar experiments were performed with the labeled oligonucleotide of the human T-cell receptor α enhancer (TCR α -GATA). This oligonucleotide contains the same AGATAG consensus sequence to which GATA-3 is known to bind (7) (Fig. 9A). The relatively low affinities of GATA-2 and GATA-3 for the G1-GATA sequence are consistent with the lack of detection of a supershift complex by anti-GATA-2 or anti-GATA-3 antiserum in ATL-16T nuclear extract.

We next addressed whether this difference in the binding affinities for G1-GATA sequence is correlated with the activity of each GATA-binding protein to transactivate the IL-5 promoter. Equal molar amounts of the expression construct (pSSR α -GATA-X) of each GATA-binding protein were cotransfected with pUC-hIL5Luc into NIH 3T3 cells. NIH 3T3 cells were chosen because the native IL-5 promoter was transactivated without PMA-A23187 stimulation. The results clearly showed that GATA-4 enhanced transcription through the native IL-5 promoter approximately eight- to ninefold relative to

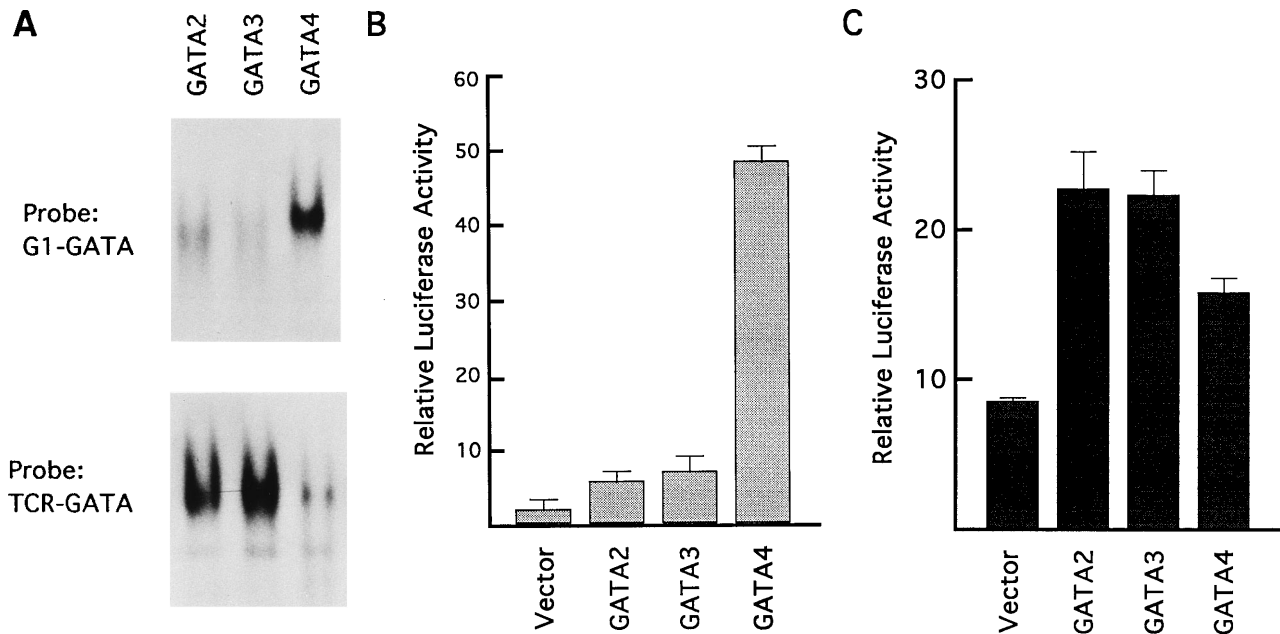


FIG. 9. GATA-4 shows a high affinity of binding to the G1 GATA site. (A) One microgram of the extract of COS cells transfected with hGATA-2, hGATA-3, or hGATA-4 expression vectors was incubated with labeled G1 (upper panel) or TCR α (lower panel) oligonucleotides and subjected to an EMSA. (B) Cotransfection of hGATA-2, hGATA-3, or hGATA-4 expression vector with pUC-hIL5Luc into NIH 3T3 cells. The transfected cells were not treated with PMA-A23187. (C) The same cotransfection experiment with p(TCR α -GATA)₄-tkLuc and Jurkat cells. The transfected cells were not treated with PMA-A23187. Vector, pSSR α without cDNA insert. Values are the means of three independent experiments, with bars representing the standard deviations of the means.

GATA-2 or GATA-3 (Fig. 9B). The effects of GATA-2 and GATA-3 were almost the same. The extent of each GATA-binding protein's transactivation of the native IL-5 promoter was correlated with the intensity of each G1-protein complex (Fig. 9, compare A [upper panel] and B). This observation was further supported by the fact that the intensity of each TCR α -GATA protein complex was again roughly correlated with each protein's transactivation of the (TCR α -GATA)₄-tk promoter construct (Fig. 9, compare A [lower panel] and C). These results indicate that among the three GATA-binding proteins, GATA-4 has the highest binding affinity for the IL-5 promoter and transactivates it most efficiently. Therefore, it is concluded that the -70 GATA element within the human IL-5 promoter is one of the target elements of the transcription factor GATA-4.

DISCUSSION

In this study, we have identified the -70 GATA element and the CLE0 regulatory element in the IL-5 proximal promoter region. Southwestern screening revealed that three candidates, GATA-2, GATA-3, and GATA-4, bind to region G1. The EMSAs and the supershift experiments revealed that GATA-4 forms a specific DNA-protein complex through interaction with the GATA site within the region G1 (Fig. 5B and 6). The effect of GATA-4, which binds to the -70 GATA site of the human IL-5 gene promoter, was examined by cotransfection experiments using the heterologous promoter or the native IL-5 promoter in luciferase reporter constructs. In the study of the heterologous promoter, the G1-GATA site was shown to be indispensable for GATA-4-mediated transactivation (Fig. 7). This effect was abolished when the mutation was introduced into the GATA site of the heterologous promoter. The result was essentially the same with the native IL-5 promoter in the presence of PMA-A23187 stimulation. The deletion or mutation of the -70 GATA element clearly abolished the transac-

tivation of the IL-5 promoter enhanced by GATA-4 (Fig. 8B and C). These results indicate that the -70 GATA site is a positive regulatory element and that GATA-4 exerts a transactivational effect through interacting with -70 GATA site.

The explanation for lack of the supershifted complexes by anti-GATA-2 and anti-GATA-3 antisera could be attributed to the low affinities of GATA-2 and GATA-3 for the -70 GATA sequence. This hypothesis was confirmed by comparing the intensities of the DNA-protein complexes formed with each recombinant GATA-binding protein and the labeled G1 oligonucleotides in EMSAs. GATA-4 gave rise to a strong shifted band while GATA-2 and GATA-3 gave weaker bands, indicating that GATA-4 has a high affinity for the -70 GATA sequence (Fig. 9A). It is surprising that the result is reversed with the TCR α -GATA sequence that includes the same recognition consensus sequence, AGATAG. This indicates that not only the binding affinities of different members of the GATA-binding family proteins but also the flanking nucleotide sequences of the consensus (A/T)GATA(G/A) motif affect the binding selectivity. The low affinities of GATA-2 and GATA-3 for the G1-GATA site are consistent with a study of the in vitro binding specificity (11). That study demonstrated that GATA-2 and GATA-3 have affinities for the underlined G of GATAG. However, our result with the TCR α -GATA sequence showed that the sequence that flanks GATAG allows GATA-2 and GATA-3 to bind to this motif in spite of the fact that this site contains a G at the fifth position (Fig. 9A).

These results support the idea that the specificity of DNA-protein interaction is determined not only by the core consensus motif but also by its flanking sequences. These observed binding preferences were further demonstrated by the cotransfection experiment using NIH 3T3 cells (Fig. 9B). The higher transactivation of the IL-5 promoter by GATA-4 compared to that by GATA-2 or GATA-3 was remarkable. Previous studies have not demonstrated such a difference in comparative co-

transfection experiments with proteins of this family (1, 5, 9, 12, 34). The result was essentially the same as in PMA-A23187-stimulated Jurkat cells, though the difference in abilities to transactivate the IL-5 promoter among the three GATA-binding proteins was not so obvious as in NIH 3T3 cells (data not shown). Therefore, this is the first study of the GATA-binding family proteins demonstrating that the DNA binding specificity accounts for the different transactivation ability for a specific promoter.

The molecular mechanism of the different binding specificity for the same recognition consensus sequence can be inferred from studies of other transcription factors. The different transactivation abilities of MRF4 and myogenin on the muscle creatinine kinase enhancer were attributed to the regions outside the DNA-binding domain (3). By analogy, therefore, the molecular mechanism of the different binding specificity for the same (A/T)GATA(G/A) sequence is possibly attributed to the nonconserved C- and N-terminal portions of the GATA-binding family proteins.

This finding may explain why the low affinities of GATA-2 and GATA-3 for the -70 GATA site resulted in the isolation of GATA-2 and GATA-3 by Southwestern screening. The nonconserved N- and C-terminal portions outside the conserved zinc finger domain could be responsible for each recognition specificity among this family of proteins, and therefore loss of these domains might have deprived the GATA-binding family proteins of their ability to discriminate each specific binding sequence. In fact, the fragments of GATA-2 and GATA-3 that were isolated by the Southwestern screening lacked the great portions of nonconserved N- and C-terminal domains. However, there is another explanation of this point. The probe we used to screen the expression library consists of six tandem repeats of the region G1 sequence. Even though the binding affinities of GATA-2 and GATA-3 for the G1-GATA site are extremely low, the maximum G1-protein interaction at six sites is possibly strong enough to give rise to positive signals in Southwestern screening. Considering that the lysogens prepared from all the three isolated clones gave rise to shifted bands with the monomeric G1 probe in an EMSA (data not shown), the former explanation seems more plausible.

We have demonstrated the major involvement of a single specific member of the family (GATA-4) in IL-5 gene regulation in a situation where three different members of the family simultaneously exist in a single cell type (ATL-16T), and therefore we have concluded that the IL-5 promoter is one of the target genes for the transcription factor GATA-4. From our study, it seems that a specific gene promoter with an (A/T)GATA(G/A) sequence is regulated selectively by one specific member of the GATA-binding family proteins. On the other hand, however, a study of the human gonadotropin α -subunit gene showed that this gene is regulated by three GATA-binding proteins, GATA-2, GATA-3, and GATA-4 (34). All of the three GATA-binding proteins were shown to bind to the human gonadotropin α -subunit gene, suggesting no selectivity among the members of the family. Similarly, the study of GATA-1⁻ embryonic stem cells suggests substantial interchangeability between GATA-1 and GATA-2 (45). From these facts, it is likely that there exist two types of gene promoters that are regulated by the GATA-binding family proteins. One is a gene that selects one specific member of the GATA-binding family proteins, like the IL-5 gene promoter, and another is a gene that is regulated by multiple members of the family. Further investigation of the functional roles of the multiple members of transcription factors with the same recognition consensus sequence will elucidate more complex mechanisms of gene regulation.

The requirement for stimulation by both GATA-4 and PMA-A23187 for the IL-5 promoter transactivation prompted us to investigate another regulatory element. We focused on the CLE0 element (-56 to -42), which is highly conserved among the promoters of several cytokines such as IL-3, IL-4, G-CSF, and GM-CSF (20, 23, 24). The study of GM-CSF CLE0 demonstrated that it mediates the PMA-A23187-induced signal and activates the GM-CSF promoter (20). We have demonstrated that the mutations introduced within the CLE0 element abolished the IL-5 promoter activity (Fig. 8C) and therefore have established its functional role in this context. The absolute requirement of the CLE0 element for induction of the IL-5 promoter may suggest that the factors interacting with this element function as components to link the upstream GATA-4 and the downstream basic transcription machinery.

It has been demonstrated that there exists a factor(s) that binds to the IL-5 CLE0 element in a PMA-A23187 stimulation-dependent manner (23). Several speculations about this factor can be made. In a study of GM-CSF CLE0, which has one base mismatch with IL-5 CLE0, AP1 and NFAT-like factors were demonstrated to bind to it (20). Another study showed that Elf-1, c-Fos, and JunB form a specific DNA-protein complex with GM-CSF CLE0 (41). We do not know whether any of these proteins participates in IL-5 CLE0-mediated transactivation of the IL-5 promoter. However, considering that the factors binding to the GM-CSF CLE0 element are also PMA-A23187 inducible, and that a highly conserved sequence motif exists in GM-CSF CLE0 and IL-5 CLE0, it is likely that the same factor or related factors are involved in IL-5 gene regulation. Further characterization of the components of IL-5 CLE0-binding factors and their relationship to GATA-4 will help to clarify the more precise mechanisms of IL-5 gene regulation.

In this study, we have clarified the possible mechanism of IL-5 gene regulation by using an ATL-derived cell line. In clinical studies, it has been reported that eosinophilia is often observed in patients with ATL (26, 40, 43). IL-5 is a cytokine that is specific for the eosinophil lineage and promotes the proliferation and differentiation of eosinophils. Our study may suggest the fundamental mechanism which leads to eosinophilia in patients with ATL via the IL-5 promoter.

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