MOLECULAR AND CELLULAR BIOLOGY, June 1999, p. 3940–3950 Vol. 19, No. 6
0270-7306/99/$04.00+0

Dimeric RFX Proteins Contribute to the Activity and Lineage Specificity of the Interleukin-5 Receptor α Promoter through Activation and Repression Domains

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Received 9 October 1998/Returned for modification 25 November 1998/Accepted 3 March 1999

Interleukin-5 (IL-5) plays a central role in the differentiation, proliferation, and functional activation of eosinophils. The specific action of IL-5 on eosinophils and hematopoietically related basophils is regulated by the restricted expression of IL-5 receptor α (IL-5Rα), a subunit of high-affinity IL-5R, on these cells. We have previously identified an enhancer-like *cis* element in the IL-5Rα promoter that is important for both full promoter function and lineage-specific activity. Here, we demonstrate by yeast one-hybrid screening that RFX2 protein specifically binds to this *cis* element. RFX2 belongs to the RFX DNA-binding protein family, the biological role of which remains obscure. Using an electrophoretic mobility shift assay, we further show that RFX1, RFX2, and RFX3 homodimers and heterodimers specifically bind to the *cis* element of the IL-5Rα promoter. The mRNA expression of RFX1, RFX2, and RFX3 was detected ubiquitously, but in transient-transfection assays, multimerized RFX binding sites in front of a basal promoter efficiently functioned in a tissue- and lineage-specific manner. To further investigate RFX functions on transcription, full-length and deletion mutants of RFX1 were targeted to DNA through fusion to the GAL4 DNA binding domain. Tissue- and lineage-specific transcriptional activation with the full-length RFX1 fusion plasmid on a reporter controlled by GAL4 binding sites was observed. Distinct activation and repression domains within the RFX1 protein were further mapped. Our findings suggest that RFX proteins are transcription factors that contribute to the activity and lineage specificity of the IL-5Rα promoter by directly binding to a target *cis* element and cooperating with other tissue- and lineage-specific cofactors.

Eosinophils, which constitute 5 to 10% of granulocytes, play an important role in host immune defense against helminthic parasites and contribute to the pathogenesis of a variety of allergic diseases associated with eosinophilia, including asthma (9, 11). Eosinophils are derived from pluripotent progenitor cells in the bone marrow, and their development and differentiation are promoted by three cytokines: interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5. Whereas GM-CSF and IL-3 play necessary roles in the proliferation of all granulocyte progenitors, including eosinophils, IL-5 is specific for eosinophil differentiation, functional activation, and survival (30, 47, 48).

IL-5 is a late-acting, lineage-specific cytokine produced primarily by activated T cells (42) and mast cells (24). The receptor for IL-5 is comprised of a unique α subunit (IL-5Rα) and a common β subunit (βc) that is common to receptors for GM-CSF, IL-3, and IL-5 (16). Low-affinity binding of IL-5 occurs with the IL-5Rα chain, and the βc chain forms a high-affinity IL-5R in combination with the IL-5Rα chain. Although signaling by the IL-5R, as well as by the receptors for IL-3 and GM-CSF, is thought to occur primarily via the βc chain, intact α and β chains are both required for optimal signal transduction (40, 41). IL-5 acts specifically on eosinophils and hematopoietically related basophils in humans (15). This lineage-specific function is regulated by the restricted expression of IL-5Rα (16). Ectopic IL-5Rα signaling has been shown to support multilineage hematopoietic development, suggesting that the main role of IL-5Rα is to restrict the action of IL-5 to eosinophils and basophils (39). Recent studies with targeted disruptions of the IL-5 and IL-5Rα genes have confirmed central and specific roles for IL-5 and IL-5Rα in regulating the development and function of eosinophils in vivo (13, 49).

Transcriptional regulation is a key step in the commitment and differentiation of each hematopoietic cell lineage (32, 43), and some of the transcription factors have been implicated in the development of eosinophils. For example, CCAAT/enhancer binding protein α (C/EBPα) is specifically upregulated during granulocytic differentiation (25). We have recently shown a selective block in granulocyte development of both eosinophils and neutrophils in C/EBPα-deficient mice (50). Both C/EBPα and C/EBPβ are reported to induce eosinophilic differentiation in a chicken hematopoietic progenitor cell line (19, 20). Transcription factors GATA-1, GATA-2, and GATA-3 are also expressed in human eosinophils (52). GATA-1 has recently been shown to directly regulate the expression of major basic protein, an eosinophil-specific granule protein (46). In spite of these findings, the molecular basis for

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the commitment of pluripotent progenitor cells to the eosinophil lineage and the transcriptional mechanisms that regulate eosinophil-specific gene expression are still poorly understood. It is clear, however, that expression of the IL-5Rα gene is a critical step in the process of eosinophil lineage commitment and differentiation, as well as in the regulation of eosinophil function. Therefore, there is considerable interest in defining the mechanisms regulating IL-5Rα gene expression and thus understanding the mechanisms regulating eosinophil development and function in general.

The RFX family of DNA-binding proteins is characterized by a highly conserved DNA binding domain and consists of five members in mammals (RFX1 to -5), two members in yeast, and one member in *Caenorhabditis elegans* (7). Among mammalian members, RFX1, RFX2, and RFX3 are closely related in structure. In contrast, RFX5 has a different structure, and only the DNA binding domain of RFX4 has been identified as a part of the variant estrogen receptor expressed in breast cancer (7). Although RFX1 has been shown to control the activity of the hepatitis B virus enhancer I (33), little is known about the cellular functions of RFX1, RFX2, and RFX3. Target genes that may be controlled by RFX1 are the ribosomal protein L30 gene (10, 29) and c-*myc* gene (26), while no potential target genes for RFX2 and RFX3 have yet been reported. On the other hand, RFX5 is well characterized as a transcriptional regulator for major histocompatibility complex (MHC) class II genes (5, 34).

In our previous analysis, we cloned the IL-5Rα promoter and identified a 34-bp upstream region that is critical for functional promoter activity (37). Further analysis identified a unique, enhancer-like *cis* element (GTTGCCTAGG) within this functional region (38). This element is important both for full promoter function and for lineage-specific activity in myeloid cells, including eosinophils. In the present study, using yeast one-hybrid screening, we determined that RFX2 directly binds to the *cis* element of the IL-5Rα promoter. Moreover, we demonstrated that RFX1, RFX2, and RFX3 bind to this element by forming either homodimers or heterodimers and activate the IL-5Rα promoter. We further showed that the ability of ubiquitous RFX proteins to activate transcription preferentially in myeloid cells is regulated through their distinct functional domains. Our findings provide a fresh insight into a regulatory mechanism of specific gene expression in eosinophils and myeloid cells in general.

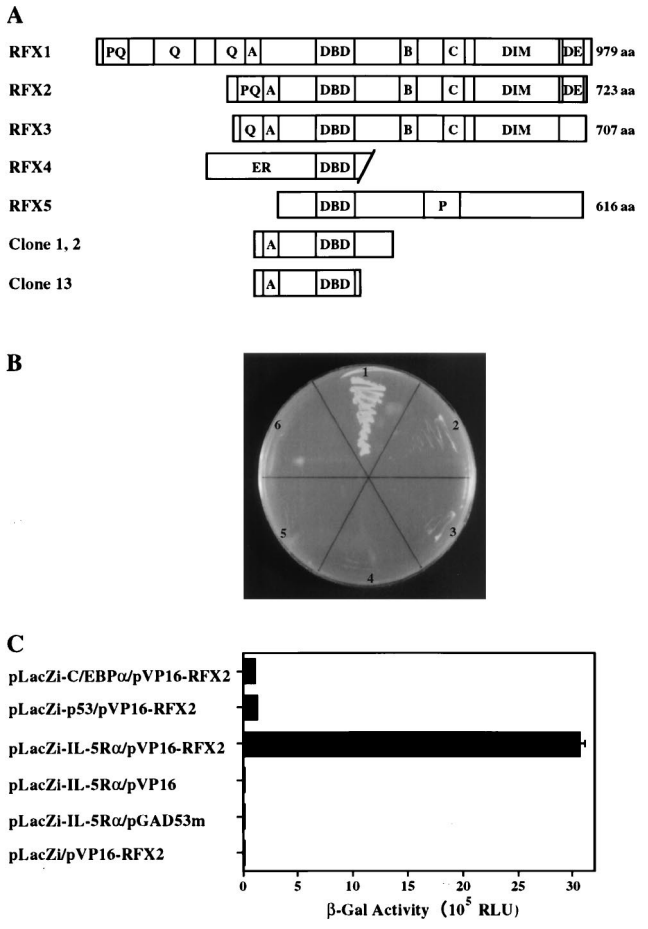


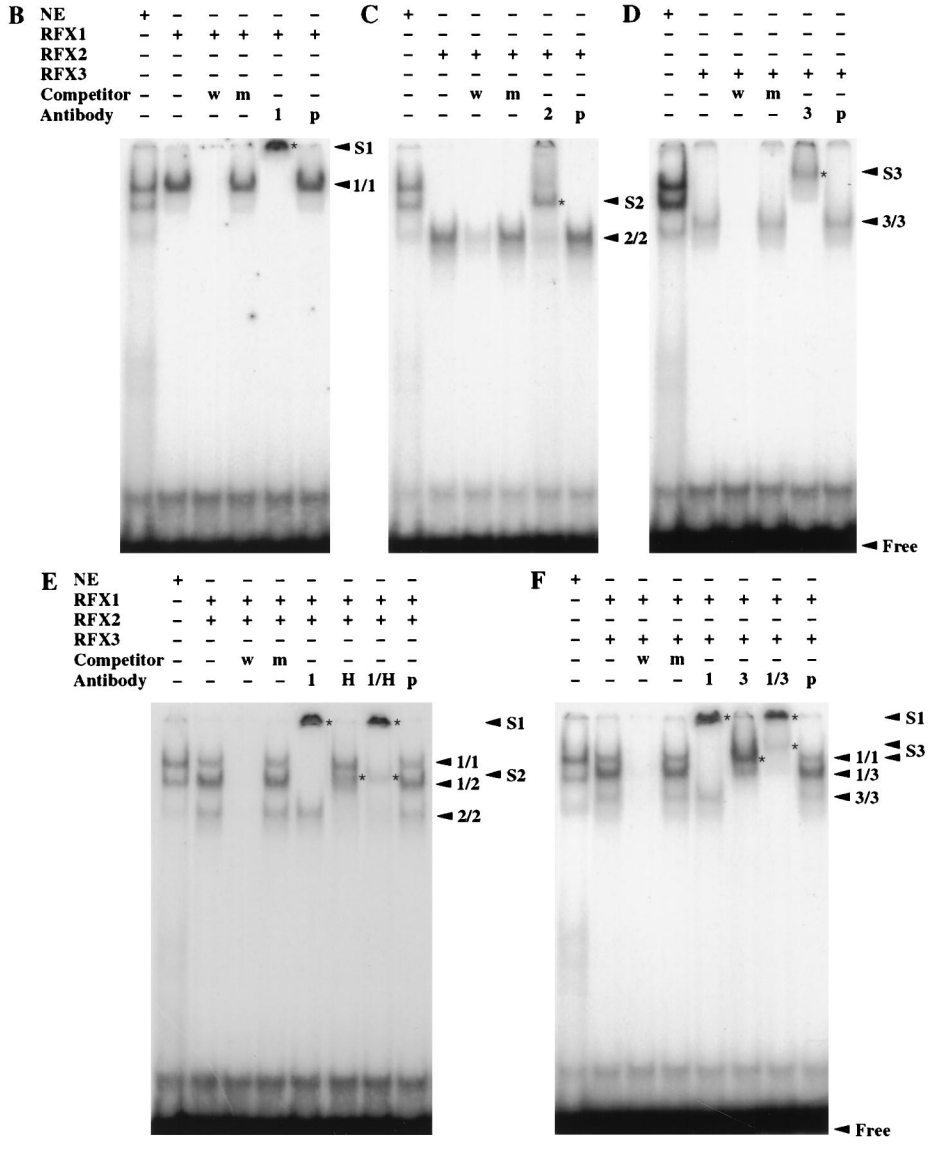
FIG. 1. Binding of RFX2 to the *cis* element of the IL-5Rα promoter in yeast. (A) Schematic representation of RFX1 to -5 (7, 28) and three RFX2 clones isolated from yeast one-hybrid screening. The DNA binding domain (DBD), dimerization domain (DIM), conserved regions A, B, and C, and estrogen receptor (ER) regions rich in proline (P), glutamine (Q), or acidic amino acids (DE) are indicated (7, 28). (B) Reporter yeast strains grown on Sabouraud dextrose plates without histidine and with 5 mM 3-amino-1,2,4-triazole at 30°C for 3 days. The reporter strain carrying pHISi-1-IL-5Rα was transformed with pVP16-RFX2 clone 1 (section 1), pVP16 (section 4), and pGAD53m (section 5). At the same time, negative-control reporter strains carrying pHISi-1-C/EBPα (section 2), pHISi-1-p53 (section 3), and pHISi-1 (section 6) were transformed with pVP16-RFX2 clone 1. (C) Binding of RFX2 to the *cis* element of the IL-5Rα promoter detected by liquid β-galactosidase assay. Bars depict β-galactosidase (β-Gal) reporter gene activity in yeast extracts from the reporter strains. The reporter strain carrying pLacZi-IL-5Rα was transformed with pVP16-RFX2 clone 1, pVP16, and pGAD53m. Negative-control reporter strains carrying pLacZi-C/EBPα, pLacZi-p53, and pLacZi were also transformed with pVP16-RFX2 clone 1. The results are shown as the means ± the standard deviations for three transformants.

MATERIALS AND METHODS

Yeast one-hybrid screening. Yeast one-hybrid screening was performed with the Matchmaker one-hybrid system (Clontech, Palo Alto, Calif.). The pHISi-1-IL-5Rα and pLacZi-IL-5Rα bait plasmids were constructed by using synthetic DNA oligomers containing a single *cis* element of the IL-5Rα promoter (bp −432 to −398). Linearized bait plasmids were integrated into the genome of the yeast YM4271 by homologous recombination. The yeast strain was then transformed with a pVP16 vector-based mouse EML cell cDNA library (14). The transformants were selected on Sabouraud dextrose plates lacking histidine and leucine and supplemented with 5 mM 3-amino-1,2,4-triazole. Selected clones were subjected to the β-galactosidase assay by using the colony-lift filter method according to the manufacturer's instructions. The liquid β-galactosidase assay was performed with a chemiluminescent substrate according to the manufacturer's instructions. Several negative-control plasmids were used in the selection procedure, including pHISi-1-C/EBPα and pLacZi-C/EBPα, containing a trimer of the *cis* element of the human C/EBPα promoter, pHISi-1-p53 and pLacZi-p53 (Clontech), containing a consensus p53 binding site, and pGAD53m (Clontech), containing the murine p53 gene fused to the GAL4 activation domain.

Cells and cell culture. An eosinophil-committed subline of the human HL-60 promyelocytic leukemia cell line, HL-60 7.7, was maintained at pH 7.7 in RPMI 1640 (Gibco BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, Mo.) and 25 mmol of *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethane-sulfonic acid (EPPS) (Sigma) per liter (45). To further differentiate HL-60 7.7 cells, butyric acid (0.5 mmol/liter) (Sigma) was added for 1 to 3 days. A human acute myeloid leukemia cell line committed to the eosinophil lineage (AML14), a human monocytic cell line (Mono Mac 6), and a human myeloblastic cell line (TF-1) were cultured and maintained as previously described (12, 23, 51). The human HepG2 hepatoma line was maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% FBS and 2 mM L-glutamine (Gibco BRL). Other human cell lines, including HL-60 (promyelocytic parental line), U937 (myelomonocytic line), THP-1 (monocytic lines), KG1a (myeloblastic line), K562 and HEL (erythroleukemic lines), Jurkat (T-lymphocytic line), Raji, BJA/B (B-lymphocytic lines), and HeLa (epithelial line), were maintained in RPMI 1640 supplemented with 10% FBS and 2 mM L-glutamine. The CV-1 monkey kidney cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Gibco BRL) and 2 mM L-glutamine. A murine lymphohematopoietic progenitor cell line (EML) was maintained in Iscove's modified Dulbecco's medium (Gibco BRL) supplemented with 20% horse serum (Gibco BRL), L-glutamine, and 10% conditioned medium from BHK cells transfected with rat stem cell factor cDNA (44). Human peripheral blood eosinophils were isolated from patients with the hypereosino-

FIG. 2. Binding of in vitro-synthesized RFX1, RFX2, and RFX3 to the *cis* element of the IL-5Rα promoter in EMSA. (A) The probe used in the gel shift assay is the *cis* element of the IL-5Rα promoter, bp −440 to −411 (37), containing a centrally located RFX binding site (8). The boxes enclose nucleotides that match the consensus sequence. R, Y, and N represent a purine, a pyrimidine, and any nucleotide, respectively. The mutant competitor was designed to contain mutations (in lowercase) of three of the six G residues on which DNA methylation interfered with nuclear factor binding in our previous study (arrows) (38). (B through F) RFX1 (B), RFX2 (C), and RFX3 (D) were individually synthesized in vitro, and RFX1 was cosynthesized with HA-tagged RFX-2 (E) or RFX3 (F); RFX-DNA complexes were analyzed by EMSA. For comparison, RFX-DNA complexes formed with AML14 nuclear extracts (NE) were loaded in the first lanes. The legends above the autoradiographs indicate the source of the in vitro translated protein, competitor, and antibody used. Antibodies 1, 2, 3, H, and p denote antibodies against RFX1, RFX2, RFX3, and HA and preimmune serum, respectively. Positions of free DNA probe (Free), RFX1 homodimers (1/1), RFX2 homodimers (2/2), RFX3 homodimers (3/3), RFX1-RFX2 heterodimers (1/2), RFX1-RFX3 heterodimers (1/3), all supershifted complexes (*), and complexes supershifted by the RFX1 antiserum (S1), the RFX3 antiserum (S3), or the RFX2 antiserum and anti-HA antibody (S2) are indicated.



philic syndrome and enriched by leukapheresis, dextran sedimentation (to remove erythrocytes), and separation over gradients of Ficoll-Hypaque (Pharmacia, Piscataway, N.J.), as previously described (52).

In vitro transcription, translation, and nuclear extract preparation. Full-length human RFX1 and RFX3 were subcloned into mammalian expression vector pSG5 (Stratagene, La Jolla, Calif.), and the resulting constructs were named pSG5RFX1 and pSG5RFX3, respectively (27, 28). Full-length human RFX2 (28) was tagged with a hemagglutinin (HA) epitope on its amino terminus and subcloned into mammalian expression vector pcDNA3 (Invitrogen, San Diego, Calif.) (pcDNA3HARFX2). RFX1, HA-tagged RFX2, and RFX3 were

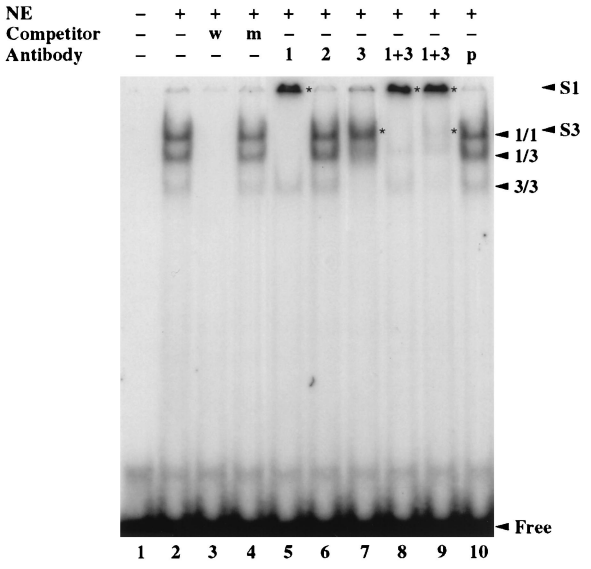


FIG. 3. Binding of native RFX protein complexes in a myeloid nuclear extract to the *cis* element of the IL-5Rα promoter in EMSA. RFX-DNA complexes were formed by using an AML14 nuclear extract and were detected by EMSA. The probe and competitors were the same as for Fig. 2. The legends above the autoradiograph indicate the source of competitor and antibody used. Abbreviations are defined in the legend to Fig. 2.

transcribed and translated in vitro by the TnT coupled reticulocyte lysate system (Promega, Madison, Wis.). Cosynthesis was performed by using mixtures of RFX plasmids at ratios of 6:1 for RFX1–HA-tagged RFX2 and 1:3 for RFX1-RFX3. Nuclear extracts were prepared from cell lines as previously described (6).

Electrophoretic mobility shift assay (EMSA). The double-stranded oligonucleotide, which encompasses the region from bp −440 to −411, was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and 0.5 ng (specific activity, 10^8 cpm/μg) per reaction was used. Proteins were incubated with 3 μg of poly(dI-dC) (Pharmacia) at room temperature for 20 min in 20 μl of 20 mM HEPES (pH 7.9), 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 3 mM $MgCl_2$, and 5% glycerol. A 50-fold molar excess of unlabeled wild-type or mutant competitor oligonucleotides was added to the binding reaction before the ^{32}P-labeled probe was added. For supershift experiments, the reaction mixtures were further incubated on ice for 20 min in the presence of either specific polyclonal antisera against RFX factors or normal rabbit serum (28). Antisera were added to the binding reaction mixture at final dilutions of 1/200 for RFX1, 1/20 for RFX2, and 1/40 for RFX3. A conditioned medium of a hybridoma producing an anti-HA antibody was used at a final dilution of 1/20. The binding reactions were then analyzed by electrophoresis at 5 V/cm for 4 h at room temperature on a native 4% polyacrylamide gel in 0.25× Tris-borate-EDTA buffer. Gels were dried and autoradiographed.

RNA preparation and Northern blot analysis. Total RNA was isolated from cell lines and human peripheral blood eosinophils by guanidium isothiocyanate extraction followed by CsCl gradient purification (4). RNA samples were resolved by agarose formaldehyde gel electrophoresis and transferred to Biotrans nylon membranes (ICN Biomedicals Inc., Costa Mesa, Calif.). The DNA fragments derived from human RFX1 (bp 1 to 832) (27), RFX2 (bp 165 to 566) (28), and RFX3 (bp 1 to 434) (28) were labeled with [α-^{32}P]dCTP and used as probes. Hybridization was performed as previously described (3). To normalize the loading of RNA samples in each lane, the blot was rehybridized to an [α-^{32}P]dCTP-labeled DNA fragment of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitation of relative mRNA levels was performed with a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, Calif.).

Construction of plasmids for reporter gene assays. Trimerized 18-mer oligonucleotides containing the RFX binding site (bp −434 to −417) with either the wild-type motif (CAGTGTTGCCTAGGAGAC) or the mutated binding motif (CAGTATTGGGTAGGAGAC) were synthesized. Double-stranded, wild-type or mutant oligonucleotides were prepared and subcloned into the enhancerless pT81 luciferase vector in front of a minimal herpes simplex thymidine kinase promoter (21). A series of deletions of the human RFX1 were generated by PCR and subcloned in frame with the GAL4 DNA binding domain in pcDNA3. All PCR product sequences were confirmed by sequencing. The enhancerless pHD luciferase reporter plasmid and pHDGAL4 luciferase containing tetramerized GAL4 binding sites were kindly provided by D. E. Ayer (1). A cytomegalovirus (CMV)-driven *Renilla* luciferase control reporter plasmid (pRL-CMV) (Promega) was used as an internal control for transfection efficiency in all transfection experiments.

Transient transfections. Suspension cells (10^7 cells/transfection) were transiently transfected by electroporation, as previously described (22, 37, 38) with minor modifications. Briefly, transfection was carried out by electroporation by using 5 μg of reporter construct, with or without 5 μg of each expression construct or empty vector, and 10 ng of pRL-CMV, with the total amount of DNA brought to 20 μg with carrier plasmid. U937 cells were electroporated at 300 V and 960 μF; HL-60 7.7, HL-60, THP-1, Jurkat, Raji, and BJA/B cells were electroporated at 250 V and 960 μF; and EML cells were electroporated at 230 V and 960 μF. The cells were harvested 5 or 24 h after transfection. Adhesion cells, including HeLa, CV-1, and HepG2, were transiently transfected by using LipofectAMINE-PLUS reagent (Gibco BRL). Samples of 3×10^4 cells were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J.) 16 h before transfection. The cells were then transfected with 200 ng of reporter construct, with or without 20 ng of each expression construct or empty vector, 200 pg of pRL-CMV, 1 μl of Lipofectamine, and 4 μl of PLUS reagent for 3 h in serum-free medium. After 3 h, serum was added to a final concentration of 10%. The cells were harvested 24 h after the beginning of transfection. Firefly luciferase activities from the reporter plasmids and *Renilla* luciferase activities from the pRL-CMV were determined with the Dual-Luciferase reporter assay system (Promega). Data are presented in relative light units (RLU) obtained by normalizing the activities of firefly luciferase to those of the *Renilla* luciferase. Individual transfection experiments were done in triplicate, and the results are reported as the means ± standard deviations from representative experiments.

RESULTS

Identification of RFX2 binding to the *cis* element of the IL-5Rα promoter. Previously, we found that a 34-bp upstream region in the IL-5Rα promoter (bp −432 to −398) was both necessary and sufficient for maximal promoter activity in vitro (37). Further characterization revealed that the region from bp −430 to −421 functioned as an enhancer-like *cis* element in myeloid cells (38). To identify the nuclear factor(s) which binds to this element, we performed yeast one-hybrid screening with a yeast strain in which two chromosomally integrated reporter genes (*HIS3* and *lacZ*) are under the control of the *cis* element of the IL-5Rα promoter (bp −432 to −398). The yeast strain was then transformed with a mouse EML hematopoietic progenitor cell library (14). The transformants were plated on selection media lacking histidine, and positive clones were subjected to a β-galactosidase assay. Four positive clones out of 2×10^6 transformants were identified by both histidine and β-galactosidase production. Sequence analysis revealed that three out of four positive clones encoded RFX2 spanning the entire DNA binding domain (Fig. 1A). The fourth clone did not contain a significant open reading frame. Retransformation experiments showed that the RFX2 clones activate *HIS3* and *lacZ* by specifically binding to the *cis* element of the IL-5Rα promoter (Fig. 1B and C).

RFX2 is a member of the RFX family of DNA-binding proteins and is closely related to RFX1 and RFX3 in structure (28) (Fig. 1A). RFX1, RFX2, and RFX3 contain a highly homologous DNA binding domain and share the same DNA binding sites, which have been identified by a site selection procedure with oligonucleotides containing a stretch of random sequence (8). DNA sequence alignment showed that the sequence from bp −430 to −417 of the IL-5Rα promoter, which is located within the bait element used in the yeast one-hybrid screening, matches the RFX binding site (Fig. 2A). This region is exactly the same region that we previously identified as a nuclear factor binding site by methylation interference analysis (38).

Specific binding of dimeric RFX1, RFX2, and RFX3 to the *cis* element of the IL-5Rα promoter. Although derived RFX2 clones without the dimerization domain presumably bind to the *cis* element of the IL-5Rα promoter as a monomer in yeast, only dimeric RFX proteins have been detected in nuclear extracts, indicating that native RFX1, RFX2, and RFX3 proteins may bind to the target sequences as homodimers or

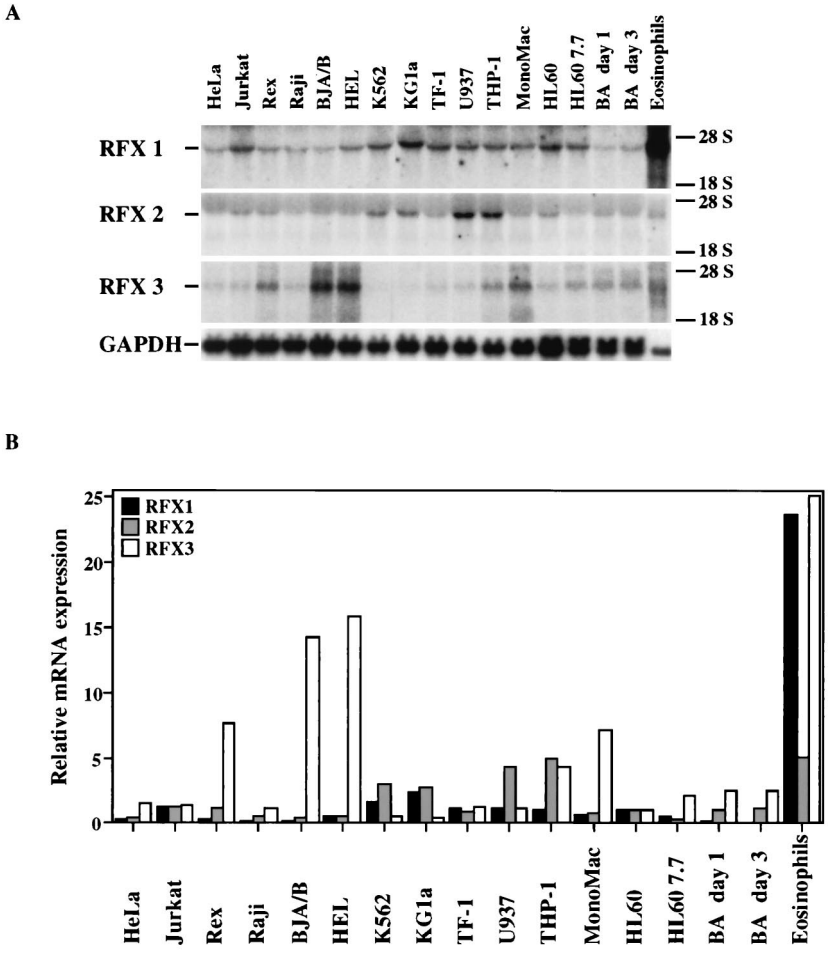


FIG. 4. Expression of RFX1, RFX2, and RFX3 in human cells. (A) Northern blot analysis of the mRNA of RFX1, RFX2, and RFX3 in human hematopoietic cells. Total RNAs from human cell lines (15 μg) and primary eosinophils (2 μg) were loaded and probed with cDNA fragments specific for human RFX1, RFX2, RFX3, or GAPDH. (B) Relative expression levels of RFX1, RFX2, and RFX3 in human cell lines and primary eosinophils. The levels of RFX mRNAs in each cell line were normalized to the levels of GAPDH mRNA and then measured relative to the levels in HL-60 cells. Evaluation of mRNA levels are consistent for each gene but cannot be compared among genes. (C) Binding of native RFX complexes in nuclear extracts from various human cell lines to the *cis* element of the IL-5Rα promoter in EMSA. RFX-DNA complexes were formed in crude nuclear extracts and were detected by EMSA. The probe and competitors were the same as for Fig. 2. Positions of RFX1 homodimers (1/1), homodimers of RFX2 or RFX3 (Homo), heterodimers of RFX1 and RFX2 or RFX3 (Hetero), and complexes supershifted by RFX1 antiserum (S1) are indicated. Free, free DNA probe.

heterodimers (28). To investigate whether dimers of RFX1, -2, and -3 could bind to the RFX consensus element of the IL-5Rα promoter, we first synthesized RFX proteins in vitro and analyzed the formation of protein-DNA complexes by EMSA. With a probe that encompasses the region from bp −440 to −411 of the IL-5Rα promoter (Fig. 2A), all homodimers of RFX1, RFX2, and RFX3 formed DNA-protein complexes (Fig. 2B to D). These complexes were competed efficiently with wild-type but not mutant oligonucleotides (Fig. 2A to D). All three complexes were supershifted with corresponding antibodies specific to RFX1, RFX2, or RFX3. These data indicated that RFX homodimers can efficiently bind to the *cis* element of the IL-5Rα promoter between bp −434 and −417 and that mutations on three G residues (at bp −430, −426, and −425) within this RFX consensus binding site fully abolish DNA binding. The weak bands that migrate faster than RFX homodimers were also competed efficiently with wild-type but not mutant competitor oligonucleotides and were supershifted with corresponding anti-RFX antibodies. They might represent the monomeric RFX complexes (Fig. 2B to F). The binding characteristics of RFX proteins as heterodimers were also analyzed by using in vitro-cosynthesized RFX1-RFX2 and

TABLE 1. Correlation between RFX binding and IL-5Rα promoter activity[a]

Construct type	Human IL-5Rα promoter construct[b]	RFX binding[c]	Transactivation[d]
WT[e]	-469 GTGTTGCCTAGGAGACAGA -414 +51	+	+++
WT[e]	-436 GTGTTGCCTAGGAGACAGA -414 +51	+	+++
Deletion[e]	-398 +51	–	–
WT[e]	-432 GTGTTGCCTAGGAGACAGA -414 +51	+	+++
Point mutation[e]	GTaTTGggTAGGAGACAGA +51	–	–
WT[e,f]	GTGTTGCCTAGGAGACAGA -377	+	++
Point mutation[e,f]	GTaTTGggTAGGAGACAGA -377	–	–
Point mutation[f]	GTGTTGCCTAccAGACAGA -377	–	–
Triplicated WT[f]	-434 3x ACGTGTTGCCTAGGAGAC -417	+	++
Triplicated mutation[f]	-434 3x ACGTaTTGggTAGGAGAC -417	–	–

[a] WT, wild type; –, no reaction; +, positive binding; ++, positive transactivation; +++, strongly positive transactivation.
[b] The G residues on which DNA methylation interfered with nuclear protein binding are indicated by arrowheads. The sequences of RFX binding sites are boxed.
[c] Binding of RFX1, -2, and -3 dimers to the consensus RFX binding site in the IL-5Rα promoter was analyzed by EMSA by using the nuclear extracts of U937 and HL-60 cells.
[d] The reporter assay was performed by using transient transfection in both U937 and HL-60 cells.
[e] Construct has been reported in our previous studies (37, 38).
[f] The fragments were subcloned upstream of the thymidine kinase minimum promoter in the pT81 luciferase vector.

RFX1-RFX3 heterodimers. Cosynthesized proteins formed protein-DNA complexes that are intermediate in mobility in addition to the two expected homodimeric complexes (Fig. 2E and F). These intermediate bands were confirmed as the RFX heterodimers by using corresponding antibodies for supershifting (Fig. 2E and F). Since anti-RFX2 antiserum was not able to supershift the RFX1-RFX2 heterodimer due to its low titer, we generated an HA-tagged RFX2 construct and used it for in vitro cosynthesis. As shown in Fig. 2E and F, the bands of intermediate mobility were supershifted with a combination of anti-RFX1 and either anti-HA or anti-RFX3 antibodies. The homodimer complexes formed by RFX1, RFX2, and RFX3 reacted only with each corresponding anti-RFX antibody (Fig. 2E and F). These data demonstrated that the RFX1, -2, and -3 homodimers and their heterodimers specifically interact with the RFX consensus element in the IL-5Rα promoter in vitro.

To further investigate whether the native RFX proteins bind to the IL-5Rα promoter, we performed EMSA with the nuclear extracts of AML14 cells, a cell line committed to the eosinophil lineage (Fig. 3). It has been shown that AML14 cells constitutively express the IL-5Rα subunit and a functional, high-affinity IL-5R (23). By using the same probe as was used for Fig. 2, protein-DNA complexes were formed in nuclear extracts and detected by EMSA. Three different complexes which comigrated with in vitro-synthesized RFX homodimers and heterodimers were formed (Fig. 2E and F and 3). As shown above, these complexes were competed efficiently with wild-type but not mutant competitor oligonucleotides. Both anti-RFX1 and anti-RFX3 antisera supershifted their corresponding bands (Fig. 3), indicating that the majority of RFX complexes in AML14 cells represent RFX1 and RFX3 homodimers and RFX1-RFX3 heterodimers. At present, however, we are not able to evaluate whether RFX2 is also involved in these complexes due to the low titer of the anti-RFX2 antiserum. Nevertheless, these results further indicated that RFX proteins directly bind to the *cis* element of the IL-5Rα promoter in vivo as either homodimers or heterodimers.

Expression of RFX1, RFX2, and RFX3 in hematopoietic cells. To detect the expression of RFX factors in hematopoietic cells, we performed Northern blot analyses with probes specific for each RFX gene. As shown in Fig. 4A, three RFX genes produced mRNAs of approximately 4.3 kb. The expression profile of RFX genes in hematopoietic cells is in good agreement with earlier observations of various tissues (28). RFX mRNAs were detected ubiquitously in various lineages of hematopoietic cells that we analyzed. However, the mRNA levels in these cells were variable. The most striking observation is that RFX1 and RFX3 genes are highly expressed in primary eosinophils. This is particularly evident for RFX1 (Fig. 4B). In addition, the expression of RFX proteins was further analyzed by EMSA with the IL-5Rα promoter as a probe (Fig. 4C). Three complexes were formed in all the cell lines which

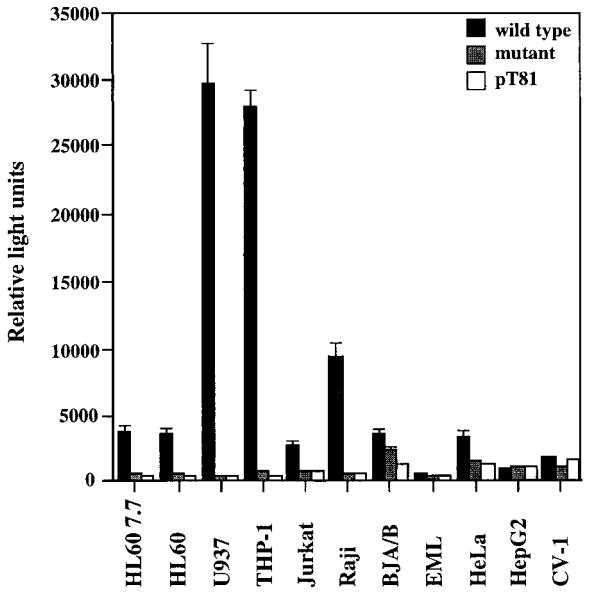


FIG. 5. Tissue- and lineage-specific enhancer activity of multimerized RFX binding sites. Trimerized RFX binding sites from the IL-5Rα promoter (bp −434 to −417), containing either wild-type or mutant sequences, were subcloned into the pT81-luc vectors in front of basal thymidine kinase promoters. The reporter plasmids and the parent pT81-luc were transiently transfected into various hematopoietic and nonhematopoietic cell lines. The minimum promoter activity of pT81-luc was approximately 300 RLU, while the background luciferase activity was below 100 RLU in all cells analyzed.

were analyzed similarly to AML14 cells. These complexes were competed efficiently with wild-type but not mutant competitor oligonucleotides (data not shown), and the upper two complexes were completely supershifted with anti-RFX1 antiserum (Fig. 4C). Among the cells that we examined, in contrast to the ubiquitous expression of RFX genes, IL-5Rα mRNA was detected only in HL-60 and its eosinophil-committed subline HL-60 7.7 at low levels, and it was strongly upregulated after 3 days of butyric acid treatment in HL-60 7.7 cells (data not shown), which is consistent with previous reports (2, 37).

Enhancer-like activity of RFX binding sites in hematopoietic cells. We have previously shown that a 38-bp region between bp −436 and −398 of the IL-5Rα promoter functions as a myeloid-specific enhancer and that the introduction of mutations within the RFX consensus sequence between bp −430 and −421, to which RFX factors were shown to bind, abolishes both DNA binding and enhancer activity (Table 1) (38). To investigate whether the enhancer activity of this region is directly mediated through the RFX factors, we constructed reporter plasmids containing a trimerized RFX binding site without any flanking elements (bp −434 to −417) in front of a basal thymidine kinase promoter (21). Reporter plasmids with either wild-type or mutated RFX binding sites and the parent enhancerless plasmid (pT81-luc) were transiently transfected into various hematopoietic and nonhematopoietic cell lines (Fig. 5). In comparison to the construct with mutated RFX binding sites, the constructs with the wild-type binding sites showed greater promoter activity, including 8-fold increases in activity in HL-60 7.7 cells (a line committed to the eosinophil lineage [45]) and parental HL-60 cells, >60-fold increases in U937 and THP-1 myeloid cells, and a 20-fold increase in Raji mature B cells (surface immunoglobulin M^+ [IgM^+], surface IgG^+). On the other hand, a fourfold increase was detected in Jurkat T cells, and less than twofold increases (or no increases at all) were detected in BJA/B immature B cells (surface IgM^+, surface IgG^-), EML murine hematopoietic progenitor cells, HepG2 hepatoma cells, HeLa cervical cancer cells, and CV-1 monkey kidney epithelial cells. These results demonstrate that the RFX binding site is critical for IL-5Rα promoter activity and indicate that RFX factors exert their enhancer activity in a tissue- and lineage-specific manner by directly binding to the RFX element of the IL-5Rα promoter.

Tissue- and lineage-specific transactivation by RFX1. It has been shown that RFX1, RFX2, and RFX3 are ubiquitously expressed. To exclude the effects of endogenous RFX proteins, we fused RFX1 to a GAL4 DNA binding domain (Fig. 6A and 7A) and analyzed its ability to activate transcription of a heterologous reporter plasmid with four GAL4 binding sites in front of a minimal promoter (pHDGAL4 luciferase). As shown in Fig. 6B, full-length RFX1 fused to a GAL4 DNA binding domain (GAL4-RFX1Full) strongly activated transcription in HL-60, U937, and THP-1 cells, in which enhancer activities of multimerized RFX binding sites have been observed (Fig. 5). In contrast, in BJA/B, HeLa, HepG2, and CV-1 cells, in which multimerized RFX binding sites were transcriptionally inactive, the GAL4-RFX1Full failed to activate transcription (Fig. 5 and 6B). To map the functional domains of RFX1 responsible for this tissue- and lineage-specific transactivation, we generated a series of 5′ and 3′ deletions fused to the GAL4 DNA binding domain (Fig. 6A and 7A). Analysis with 5′ successive deletions showed that the region spanning the DNA binding domain and the carboxy terminus (residues 416 to 979) does not transactivate in either U937 or HeLa cells (Fig. 6C). We next analyzed 3′ deletions of RFX1 fused to the GAL4 DNA binding domain (Fig. 7A). The amino-terminal domain of RFX (GAL4-RFX475 and GAL4-RFX620) showed a similar transactivation ability in all cell types that were tested, including myeloid, nonmyeloid, and nonhematopoietic cells (Fig. 7B and data not shown), suggesting that this region contains the activation domain. However, this activation potential was completely masked by adding residues 621 to 739, a region which covers domains B and C (Fig. 7B), indicating the existence of an inhibitory domain. In addition, deletion of the carboxy terminus from residues 909 to 979 fully abolished tissue- and lineage-specific transactivation by full-length RFX1 (Fig. 7B).

DISCUSSION

It is still unclear how human cytokine and growth factor receptor genes are regulated during the commitment and differentiation of hematopoietic progenitors to the myeloid lineage in general or to the eosinophil lineage in particular. IL-5 is a late-acting, lineage-specific cytokine which functions on cells of eosinophil lineage (30). The specific action of IL-5 is mediated through binding to the unique subunit of its receptor, IL-5Rα (41). Like other hematopoietic growth factor receptors, IL-5Rα plays a critical role in the process of differentiation myeloid progenitors into particular eosinophilic developmental programs. Therefore, it is extremely important to understand the regulation process of IL-5Rα gene expression.

In our previous studies, we identified an enhancer-like element within the functionally active 34-bp region of the IL-5Rα promoter. This element was further narrowed down to a 10-bp region between bp −430 and −421 by methylation interference analysis (Table 1) (38). Based on these results, we inserted the 34-bp element of the IL-5Rα promoter as a bait element in front of a minimum promoter of both *HIS* and *lacZ* reporter plasmids. We successfully isolated three RFX2 clones from 2×10^6 transformants. Although it has been suggested that at

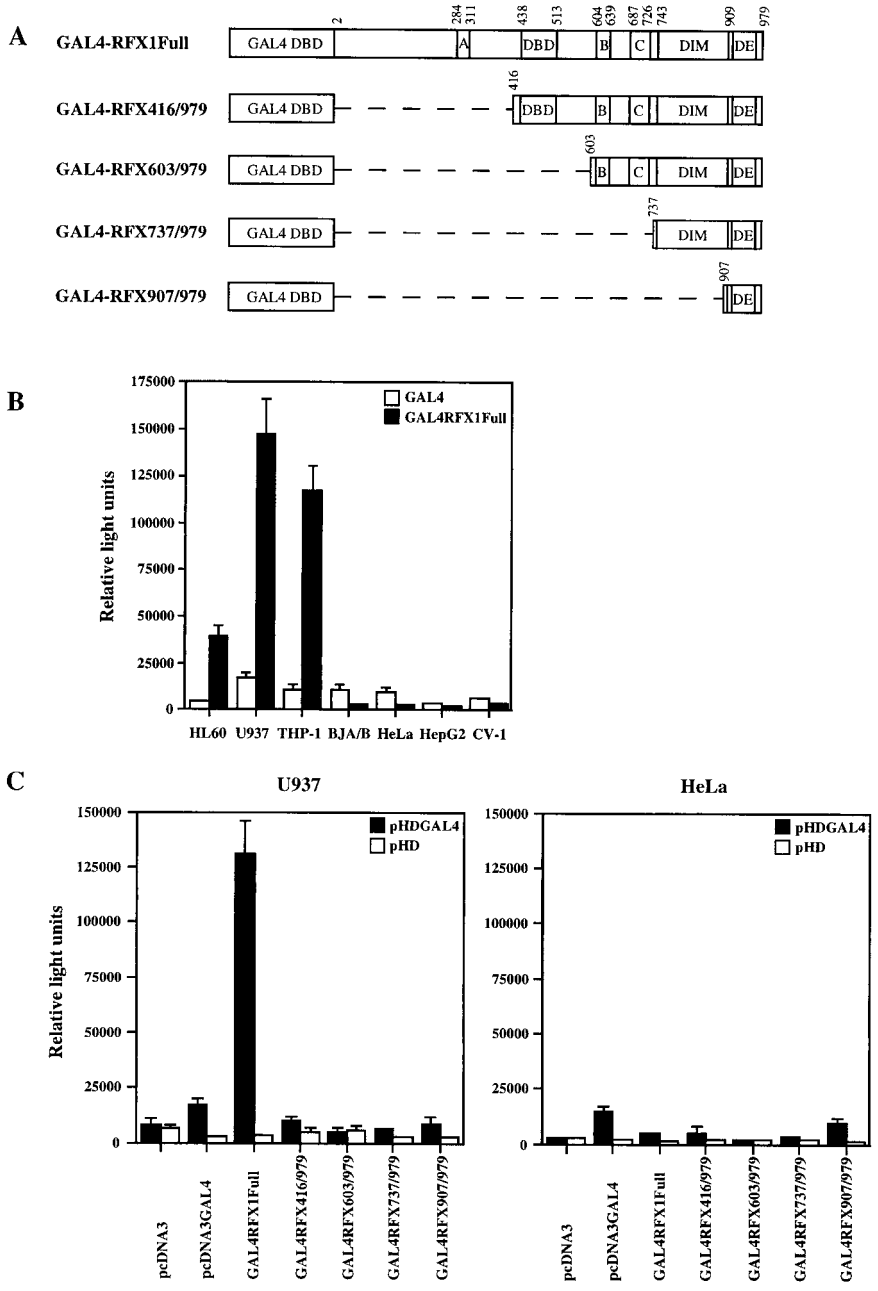


FIG. 6. Tissue- and lineage-specific transactivation by GAL4-RFX1 fusion proteins. (A) Schematic representation of RFX1 and its 5′ deletions fused to the GAL4 DNA binding domain (DBD). The numbers correspond to amino acid residues of each domain and the deletion sites (dashed lines). DE, acidic amino acids; DIM, dimerization domain. (B) A reporter plasmid containing multimerized GAL4 binding sites (pHDGAL4 luciferase) or its parent pHD luciferase plasmid was transiently cotransfected with either pcDNA3 containing the GAL4 DNA binding domain only (GAL4) or full-length RFX1 fused to the GAL4 DNA binding domain (GAL4-RFX1Full) into various cell lines. The lowest luciferase activity was obtained in HepG2 (2,360 RLU), while the background luciferase activity was below 100 RLU in all cells analyzed. (C) Mapping of RFX1 regions that activate transcription. A series of 5′ deletion mutants of RFX1 fused to the GAL4 DNA binding domain were cotransfected with the reporter plasmid into U937 and HeLa cells. The minimum promoter activity of pHD luciferase plasmid was 6,053 RLU in U937 cells and 1,556 RLU in HeLa cells, while the background luciferase activity was 42 RLU in U937 cells and 85 RLU in HeLa cells.

least three tandem copies of the target element are required to activate reporter genes, we have successfully isolated a binding protein by using a single copy of the target element, indicating that the multiple elements may not be critical. Instead, to determine and use a specific target DNA fragment may be a key in yeast one-hybrid screening. It is currently unclear why only RFX2 clones were isolated in our screening, but it seems to be due to the higher number of RFX2 cDNA clones in this particular EML library.

DNA sequence alignment showed that the element from bp −430 to −417 of the IL-5Rα promoter matches the consensus RFX binding site (Fig. 2A and Table 1), which is exactly the same region that we previously identified as a nuclear factor binding site by methylation interference analysis (38). The

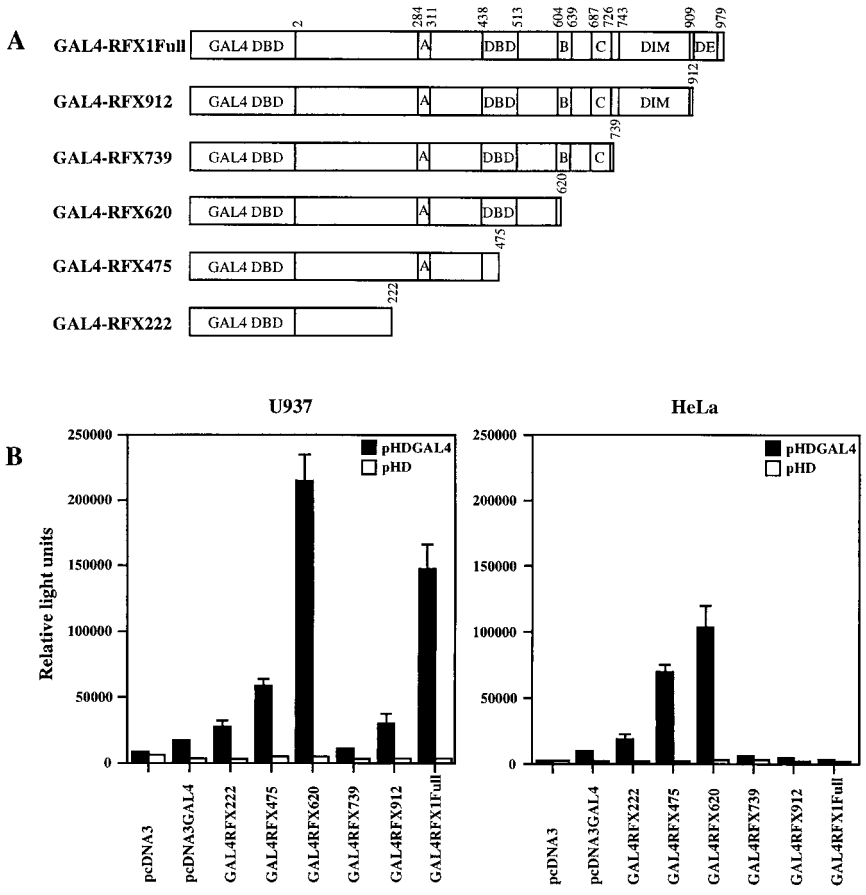


FIG. 7. Localization of the activation and inhibitory domains of RFX1. (A) Schematic representation of RFX1 and its 3′ deletions fused to the GAL4 DNA binding domain (DBD). Numbers correspond to amino acid residues of each domain; DIM, dimerization domain; DE, acidic amino acids. (B) A series of 3′ deletion mutants of RFX1 fused to the GAL4 DNA binding domain were cotransfected with the reporter plasmid into U937 and HeLa cells. The minimum promoter activity of pHD luciferase plasmid was 6,053 RLU in U937 cells and 1,556 RLU in HeLa cells, while the background luciferase activity was 42 RLU in U937 cells and 85 RLU in HeLa cells.

formation of two DNA-protein complexes (C1 and C2) was observed in nuclear extracts in our previous experiments. Their identical methylation interference patterns suggested that these complexes were formed by a nuclear factor or factors with the same DNA-binding properties (38). This previous prediction was confirmed by our present results from supershift experiments. With the specific RFX antibodies, we determined that C1 and C2 complexes represent RFX1 homodimers and RFX1-RFX3 heterodimers, respectively, which is consistent with the result of Northern blot analysis showing predominant expression of RFX1 and RFX3 mRNAs in primary eosinophils. Interestingly, an RFX consensus binding site was also identified in the upstream region of the mouse IL-5Rα promoter by a database search, further indicating the importance of this element.

Although earlier studies have shown that RFX1 is involved in the regulation of the hepatitis B virus enhancer I (33), no cellular targets for RFX1, RFX2, and RFX3 had been defined. In this report, we have provided several lines of evidence to show IL-5Rα to be the first cellular target regulated by RFX1, RFX2, and RFX3. Point mutation and deletion analyses of the IL-5Rα promoter clearly demonstrated that the nucleotides required for IL-5Rα promoter activity correspond precisely to the nucleotides required for RFX protein binding (Table 1). Moreover, transactivation of the IL-5Rα promoter through the RFX element was observed preferentially in myeloid cells. All these findings suggest that RFX proteins play a central role in controlling IL-5Rα gene expression in myeloid cells. To further address the role of RFX in the regulation of IL-5Rα gene expression, we introduced three potential dominant negative mutants of RFX1, which included the DNA binding domain (amino acids [aa] 416 to 620), C terminus (aa 416 to 979), and N terminus (aa 2 to 739), into butyric acid-treated HL-60 7.7 cells by transient transfection. Preliminary experiments showed approximately 1.4- and 1.7-fold declines in the levels of IL-5Rα mRNA in the cells transfected with the C-terminal and N-terminal constructs, respectively (data not shown). This result supports a central role for RFX proteins in the regulation of IL-5Rα expression. However, IL-5Rα mRNA was detected in some, but not all, myeloid lines in which the RFX element is active. This suggests that other transcription factors are also involved in the regulation of IL-5Rα gene expression. In addition to being active in myeloid cells, the enhancer activity of the RFX element was also active in Raji cells (mature B cells), consistent with the fact that the IL-5Rα gene is expressed in B cells in mice (49).

Our data and those of others have shown that RFX1, RFX2, and RFX3 are ubiquitously expressed. The RFX proteins were also detected by EMSA in BJA/B, EML, HeLa, and CV-1 cells, in which the RFX element is not transcriptionally active.

Moreover, the overexpression of RFX proteins in these cells was not able to induce significant enhancement of transcriptional activity mediated by multimerized RFX elements in vitro or induce expression of the IL-5Rα gene in vivo (data not shown). These results strongly suggest that additional factors are required to cooperate with RFX proteins in controlling IL-5Rα gene expression. With the GAL4-RFX1Full construct, we provided experimental evidence that suggests the involvement of lineage-specific cofactors which modulate RFX function. Several functional domains of RFX1 were mapped by using the GAL4-DBD system (Fig. 6 and 7). The amino-terminal domain (residues 1 to 438) functioned as a transactivation domain and activated transcription in all types of cells. A repression domain located around domains B and C showed a common repressive effect on transcription. This domain was able to mask the transactivation mediated by the amino-terminal domain. However, this repression effect could be overcome in a tissue- and lineage-specific manner with full-length RFX1. The carboxy-terminal region (residues 909 to 979) appeared to be important in this process (Fig. 7B). This region is not able to activate transcription independently; however, it showed a coactivation effect with the amino-terminal domain. It is conceivable that this region interacts with lineage-specific cofactors independently or cooperatively with the activation domain to modulate transcription.

Recent studies of another RFX protein, RFX5, provide a useful model for us to characterize RFX1 function. RFX5 has a DNA binding domain highly characteristic of the RFX family and specifically recognizes a DNA element unique to MHC class II genes (5, 7, 34). Like other RFX members, RFX5 is ubiquitously expressed. However, it has been demonstrated that transactivation mediated by RFX5 fully relies on a coactivator, CIITA, the expression of which is restricted to dendritic and B cells and is inducible by gamma interferon in a variety of other cell types (17, 18, 35, 36). RFX5 and CIITA are believed to associate to form a protein complex which is capable of activating transcription from promoters of MHC class II genes (31). The data in this study suggest that RFX1 may also function through interaction with other lineage-specific cofactors.

The respective roles of RFX1, RFX2, and RFX3 still remain obscure. As shown in Fig. 1A, they share homologous structures, except for the longer amino terminus of RFX1, and they share the same DNA binding specificity. In addition, the repression domain of RFX1 is localized to the region containing domains B and C, which are highly conserved among these three RFX proteins. These findings are indicative of a redundant function for RFX factors. However, we do not know whether secondary structures formed by each homodimer and heterodimer vary and, therefore, cooperate differently with other proteins. The identification of cofactors associated with RFX1, -2, and -3 proteins is important to further understand the cooperative protein-protein interactions that are required for the transcriptional regulation of the IL-5Rα gene and other myeloid genes as well.

ACKNOWLEDGMENTS

We thank S. Tsai for providing the EML cell library, EML cells, and BHK-MKL cells, R. P. de Groot for providing HL-60 7.7 cells (45), D. E. Ayer for providing pHDluciferase and pHDGAL4luciferase (1), B. Müller and S. Narravula for their excellent technical support, and L. K. Clayton and L. M. Johansen for critical reading of the manuscript.

This work was supported by National Institutes of Health grants CA70297 (to Z.S.) and CA41456 (to D.G.T.), American Cancer Society grant RPG98213 (to Z.S.), and fellowship awards from the Japan Research Foundation for Clinical Pharmacology and the Human Frontier Science Program (to A.I.).

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