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Functional Domains and DNA-binding Sequences of RFLAT-1/KLF13, a Krüppel-like Transcription Factor of Activated T Lymphocytes*

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RFLAT-1/KLF13, a member of the Krüppel-like family of transcription factors, was identified as a transcription factor expressed 3–5 days after T lymphocyte activation. It binds to the promoter of the chemokine gene RANTES (regulated on activation normal T cell expressed and secreted) and regulates its "late" expression in activated T-cells. In this study, a series of experiments to define the functional domains of RFLAT-1/KLF13 were undertaken to further advance the understanding of the molecular mechanisms underlying transcriptional regulation by this factor. Using the GAL4 fusion system, distinct transcriptional activation and repression domains were identified. The RFLAT-1 minimum activation domain is localized to amino acids 1–35, whereas the repression domain resides in amino acids 67–168. Deletion analysis on the RFLAT-1 protein further supports these domain functions. The RFLAT-1 activation domain is similar to that of its closest family member, basic transcription element-binding protein 1. This domain is highly hydrophobic, and site-directed mutagenesis demonstrated that both negatively charged and hydrophobic residues are important for transactivation. The nuclear localization signal of RFLAT-1 was also identified using the RFLAT-1/green fluorescence protein fusion approach. RFLAT-1 contains two potent, independent nuclear localization signals; one is immediately upstream of the zinc finger DNA-binding domain, and the other is within the zinc fingers. Using mutational analysis, we also determined that the critical binding sequence of RFLAT-1 is CTCCC. The intact CTCCC box on the *RANTES* promoter is necessary for RFLAT-1-mediated *RANTES* transcription and is also required for the synergy between RFLAT-1 and NF-κB proteins.

Transcription factors are sequence-specific DNA-binding proteins that regulate gene expression by either activating or repressing the initiation of transcription. Mammalian transcription factors are grouped into several categories based upon their structural domains for DNA binding and include the helix-loop-helix, homeodomain, leucine zipper, and zinc finger protein families (1). The zinc finger proteins are further classified based on the number of zinc fingers and the amino acid residues responsible for zinc binding. Among them, the Cys_2His_2 (C_2H_2)[1] zinc finger proteins have been estimated to make up 1% of the human genome (2). The majority of these have not been characterized, and their biological functions are just beginning to be elucidated. A subset of C_2H_2 proteins contains a DNA-binding domain consisting of three contiguous C_2H_2 zinc fingers at the carboxyl terminus. Because their highly conserved DNA binding motifs resemble those of the segmentation gene product of *Drosophila melanogaster*, Krüppel, they have been named Krüppel-like factors (KLFs) (3, 4). Based on a recent BLAST search, this rapidly growing family now has 19 members and includes one of the first identified general transcription factors, Sp1 (5). These KLFs play critical roles in regulating a diverse range of biological processes, including cell growth, differentiation, embryogenesis, and tumorigenesis (6).

We have been investigating the transcriptional control of RANTES gene expression to understand the "late" expression kinetics of *RANTES* in activated T lymphocytes. RFLAT-1 was identified by expression cloning through its binding to the A site of the *RANTES* promoter (7). In addition to activating the RANTES gene in T-cells, RFLAT-1 (also known as FKLF2 and BTEB3) can activate the human γ globin promoter, other erythroid-specific genes, SV40, and SM22α promoters (8, 9). DNA binding studies demonstrate that RFLAT-1 bound to the A site of the *RANTES* promoter, a consensus basic transcription element (BTE), and CACCC box of the γ globin promoter (7–9).

By sequence analysis, RFLAT-1 belongs to the KLF family and shares the greatest homology with basic transcription element-binding protein 1 (BTEB1/KLF9) and BTEB4 (see Fig. 1). Although the amino acid sequences in the zinc finger domains of KLFs are highly conserved and bind to similar DNA sequences, the regions outside of the zinc fingers are not

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[1] The abbreviations used are: C_2H_2, Cys_2His_2; RANTES, regulated upon activation, normal T-cell expressed and secreted; RFLAT, RANTES factor of late activated T lymphocytes; KLF, Krüppel-like factor; BTE, basic transcription element; BTEB, BTE-binding protein; NLS, nuclear localization signal(s); GFP, green fluorescence protein; DBD, DNA-binding domain; EMSA, electrophoretic mobility shift assay; AA, amino acid(s); DRRF, dopamine receptor-regulating factor; CBP, cAMP-response element-binding protein-binding protein.

homologous. This heterogeneity may in part account for the tissue-specific expression of KLFs, the diverse biochemical mechanisms by which KLFs function, and explain their specific roles in a variety of biological processes. The structure-function relationships of selected KLFs have been studied in detail. Sp1, a relatively large protein (782 amino acids), contains multiple transactivation domains, including two glutamine-rich activation motifs and Ser/Thr rich regions (10). Although Sp1 is a potent activator of transcription, some KLFs, like Sp3 (11) and KLF12 (12), are repressors. Other KLFs, such as EKLF/KLF1, GKLF/KLF4, and LKLF/KLF2, contain separate transcriptional activation and repression domains (13–17). The transactivation domain of EKLF/KLF1 is critical for cell-specific inducibility of a β-globin promoter (18). GKLF/KLF4 activates the human keratin 4 and Epstein-Barr virus ED-L2 promoters (19), whereas it suppresses the activity of the CYP1A1 promoter (20). Thus, despite similar DNA binding properties, the specific biological activities of KLFs may largely rely on their distinct domains outside of DNA binding, and characterization of these motifs may help elucidate their functions.

The human RFLAT-1/KLF13 is a 288-AA polypeptide with three C_2H_2 fingers at the carboxyl terminus (AA 169–249). The zinc finger domain is responsible for DNA binding and also appears to mediate interaction with coactivators cAMP-response element-binding protein-binding protein/p300 and p300/CBP-associated factor (21). Nevertheless, little is known about the function of other structural regions of RFLAT-1/KLF13. The present study is a detailed analysis of selected functional domains of RFLAT-1/KLF13. We show that RFLAT-1 contains distinct transcriptional activation and inhibitory domains, which reside at the very end of the amino terminus and the middle portion of the protein, respectively. The activation domain, which differs from any of the well known transactivation motifs, is rich in hydrophobic amino acids and embedded with negatively charged residues. These negatively charged residues are important for transactivation activity. We identified two potent, independent NLS within RFLAT-1, either of which is sufficient to translocate GFP into the nucleus. We also demonstrate that RFLAT-1 recognizes and binds to a CTCCC box within the *RANTES* promoter. The intact binding sequence is absolutely required for RFLAT-1-mediated *RANTES* transcription in T-cells. Finally, the structural similarities and differences between RFLAT-1/KLF13 and other KLFs are compared to better specify their biological functions.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Truncated *RFLAT-1* cDNAs were generated by PCR with Advantage® cDNA PCR kit (CLONTECH) and corresponding 5′- and 3′-primers (PCR primer sequences available upon request). The PCR-generated DNA fragments containing appropriate start codon, Kozak sequence, and stop codon were subcloned into a mammalian expression vector pcDNA3.1/V5-His TOPO using a TA cloning kit (Invitrogen). GAL4-RFLAT-1 chimeric constructs were made by fusing RFLAT-1 fragments in-frame to the carboxyl terminus of the GAL4 DNA-binding domain (DBD; amino acids 1–147). For this purpose, segments of RFLAT-1 were generated by PCR, and the PCR products were then digested with *Xba*I and *Kpn*I, separated on a 1.5% agarose gel, purified using a Concert kit (Invitrogen), and ligated into the corresponding sites of the cytomegalovirus-driven mammalian expression vector pBIND (Promega), which contained the GAL4 DBD. Site-directed mutagenesis was used to mutate the indicated amino acids within the GAL4-RFLAT-1 1–35 construct. D8A, E32A/S33A mutants and *RANTES* promoter mutants were generated in the same manner as the deletion constructs. The other mutants were made using a modified version of megaprimer mutagenesis PCR as described (22). For GFP fusion constructs, the PCR products encoding fragments of *RFLAT-1* cDNA were subcloned into a mammalian expression vector pcDNA3.1/CT GFP (Invitrogen). For all of the constructs, the PCR procedure consisted of heating at 95 °C for 5 min, 30 cycles of 95 °C for 30 s and 68 °C for 3 min, and finally incubating at 68 °C for 3 min. The reading frame and sequences of all of the constructs were confirmed by DNA sequence analysis.

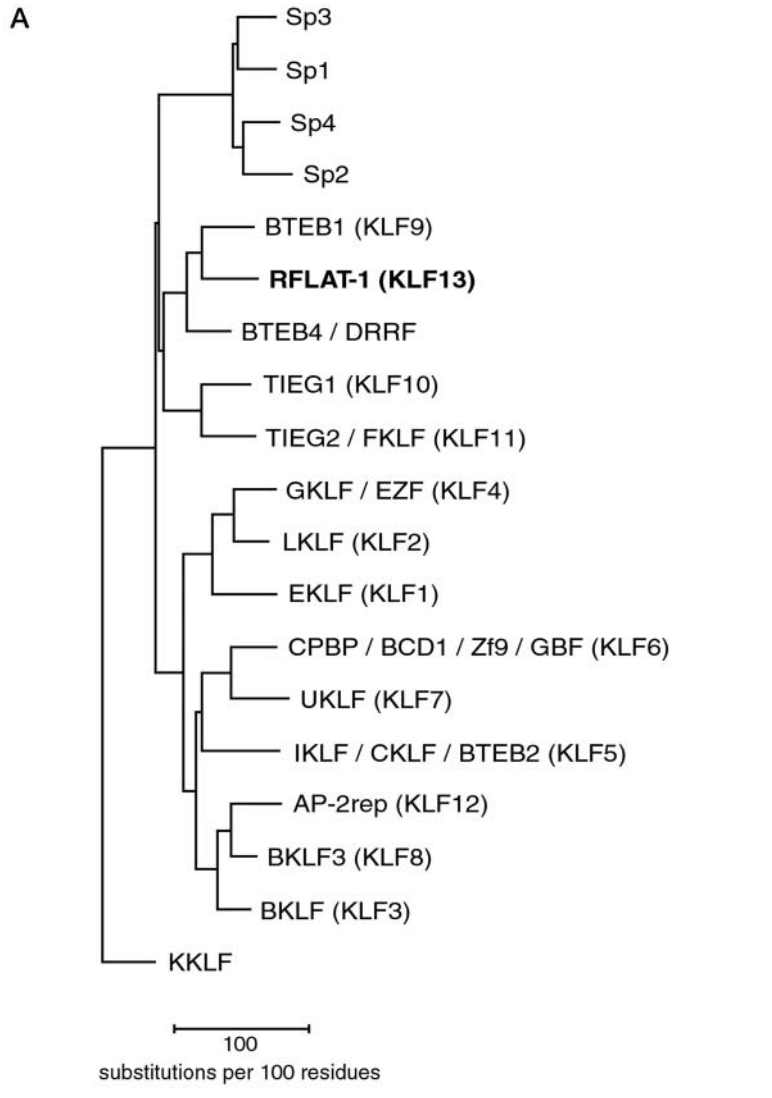
Cell Culture, Transfections, and Cellular Localization of GFP Fusion Proteins—Fibroblast cells NIH 3T3, COS-7, and Jurkat T tumor cells were cultured in either Dulbecco's modified Eagle's medium (Invitrogen), or RPMI 1640 medium (Irvine Scientific) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The fibroblasts (10^6 cells/60-mm dish) were transiently transfected by either the calcium phosphate method (7) or the LipofectAMINE procedure (Invitrogen) according to the manufacturer's instructions. Jurkat T-cells were transfected by electroporation as described previously (23). For GAL4 fusion experiments, the reporter plasmid was pG5*luc* (Promega), which contained five GAL4-binding sites upstream of the luciferase gene. The expression plasmid pBIND also contained a *Renilla* luciferase gene downstream of the fusion protein that allowed normalization for transfection efficiency. When pcDNA3.1-derived expression plasmids were cotransfected with *RANTES* promoter-driven luciferase reporter gene pGL_2R (7), a pRL-null (Promega) plasmid encoding a *Renilla* luciferase gene was included for normalization. 36–48 h after transfection, the cells were harvested, and luciferase activity was determined using a dual luciferase assay system kit (Promega) following the manufacturer's instructions. Luciferase activity was measured in a Wallac/EG&G Lumat LB 9507 Luminometer. To visualize RFLAT-1-GFP fusion proteins, the fusion constructs were transfected using the FuGENE method (Roche Molecular Biochemicals) into COS-7 cells grown on tissue culture glass slides. 48 h later, the cells were treated with MitoTracker Red (Molecular Probes) for 15 min and subsequently fixed with 4% paraformaldehyde. Vectashield with DAPI mounting medium (Vector Laboratories) was used to counterstain DNA and preserve fluorescence. The cellular localization of various fusion proteins was monitored using a Nikon Eclipse E800 microscope equipped for epifluorescence.

Immunoblotting Assay and Production of Recombinant RFLAT-1—The level of protein expression by transfected constructs was determined by immunoblotting. After the cells were transfected as described above, nuclear extracts were prepared according to Andrews and Faller (24), and protein concentration was determined by Bradford assay. The proteins were separated on an SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk overnight at 4 °C and probed with either a polyclonal anti-GAL4 (1–147) antibody (Santa Cruz Biotechnology) or a protein A column purified polyclonal anti-RFLAT-1 antibody. They were further incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody and visualized by ECL (Amersham Biosciences). The full-length recombinant RFLAT-1 protein was produced by subcloning the human *RFLAT-1* cDNA into a pET-28a(+) vector (Invitrogen). The expression and purification of the His-tagged RFLAT-1 through a Ni^+ column were described previously (7).

Electrophoretic Mobility Shift Assay—Oligonucleotides used for EMSA were synthesized and PAGE-purified by Invitrogen. The oligonucleotides were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). A typical binding reaction mixture contained 15,000–20,000 cpm of probe, 2–8 μg of nuclear extracts or recombinant protein, 1.5 μg of poly(dI-dC)·poly(dI-dC), 10 mM Tris-HCl, pH 7.5, 80 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol. The mixture was incubated at 4 °C for 30 min and fractionated on a 8% nondenaturing polyacrylamide gel in 1× Tris-borate-EDTA buffer.

RESULTS

RFLAT-1/KLF13 Is a Member of the KLF Family with the Closest Similarity to BTEB1 and BTEB4—A phylogenetic tree was generated to reveal the structural relationships between RFLAT-1/KLF13 and 18 other human KLFs identified in a recent BLAST search (Fig. 1*A*). RFLAT-1, BTEB1, and BTEB4 form a separate subfamily that is relatively distant from other well studied KLFs, such as Sp1 and EKLF/KLF1. By sequence comparison, the homology of RFLAT-1/KLF13 with the other KLFs is limited to the zinc finger DNA-binding domain. Similarity outside of the zinc fingers is only found among RFLAT-1, BTEB1, and BTEB4 (Fig. 1*B*). Overall, at the protein level, RFLAT-1 is 41% identical to BTEB1 and 43% identical to BTEB4. The sizes of the three proteins are also similar (RFLAT-1, 288 AA; BTEB1, 244 AA; and BTEB4, 252 AA) compared with the relatively larger sizes of other KLFs (EKLF,



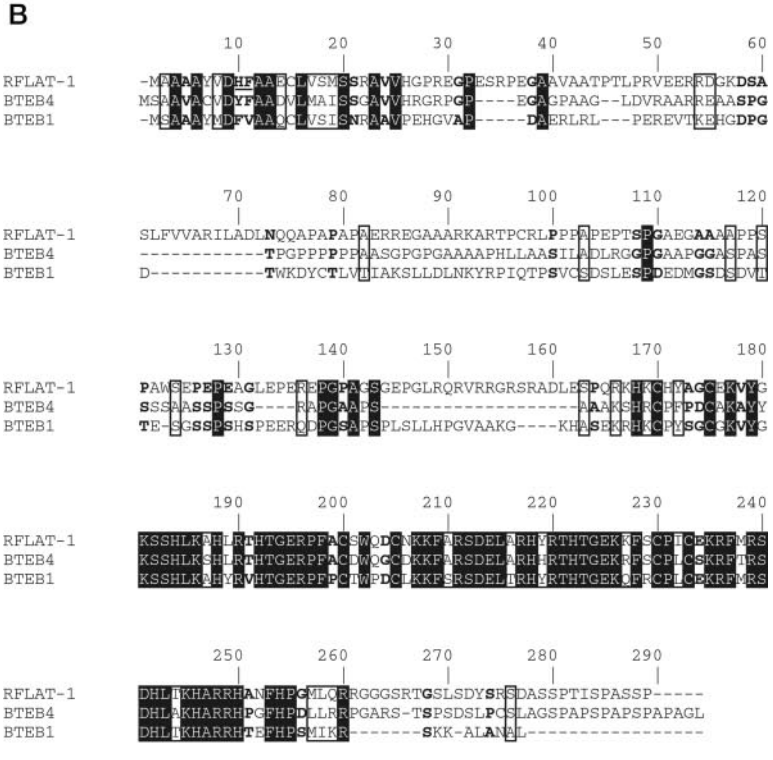


FIG. 1. **Structural similarities of RFLAT-1/KLF13 with other Krüppel-like factors.** *A*, human KLFs were identified by BLAST search using human RFLAT-1/KLF13 protein sequence (GenBank™ accession number AAD26846). The phylogenetic tree was generated by the Kimura algorithm using the GCG SeqWeb GrowTree evolutionary analysis program. The *horizontal bar* indicates 100 amino acid changes. The accession numbers for each protein are as follows: Sp3, Q02447; Sp1, AF252284; Sp4, Q02446; Sp2, Q02086; BTEB1, Q13886; BTEB4, AF327440; RFLAT-1, AAD26864; TIEG1, Q13118; TIEG2, O14901; GKLF/EZF, O43474; LKLF, Q9y5W3; EKLF, Q13351; CPBP/BCD1/Zf9/GBF, Q99612; UKLF, O75840; IKLF/CKLF/BTEB2, Q13887; AP-2rep, CAB46982; BKLF3, NP_009181; BKLF, P57682; and KKLF, BAA88561. The abbreviations are as follows: *TIEG/FKLF*, transforming growth factor-β-inducible early growth response/fetal and embryonic Krüppel-like factor; *GKLF/EZF*, gut-enriched Krüppel-like factor/epithelial zinc finger; *LKLF*, lung Krüppel-like factor; *EKLF*, erythroid Krüppel-like factor; *CPBP/BCD1/Zf9/GBF*, core promoter-binding protein/B-cell derived 1/zinc finger 9/GC-rich sites binding factor; *UKLF*, ubiquitous Krüppel-like factor; *IKLF/CKLF/BTEB2*, intestinal-enriched Krüppel-like factor/colon Krüppel-like factor/basic transcription element-binding protein 2; *AP-2rep*, AP-2 repressor; BKLF: basic Krüppel-like factor; and *KKLF*, kidney Krüppel-like factor. BTEB4 and KKLF have not received a nomenclature from the Human Gene Nomenclature Committee. *B*, amino acid sequence alignment of human RFLAT-1, BTEB4, and BTEB1. The multiple alignment was performed by Clustal W. Identical, strongly similar, and weakly similar amino acids are indicated by a *black box*, a *white box*, or *bold type*, respectively. The *dashes* indicate missing amino acids.

362 AA; GKLF, 470 AA; TIEG2, 512 AA, etc.) (6). These data suggest that these three proteins may be more closely related during evolution and may represent a subfamily with similar characteristics.

RFLAT-1/KLF13 Contains Distinct Transcriptional Activation and Repression Domains—Sequence analysis of RFLAT-1/KLF13 revealed a DNA-binding domain (AA 169–249) with three typical contiguous C_2H_2 zinc fingers, a serine-rich carboxyl-terminal tail (AA 250–288), a basic region adjacent to the amino terminus of zinc fingers (AA 147–168), and an amino-terminal domain (AA 1–146) rich in proline (24/146), serine (10/146), and alanine (30/146) residues, which are known to constitute transcriptional activation domains for a number of transcription factors (1). We hypothesized that the RFLAT-1/KLF13 transactivation domain may reside within this region. To investigate this, the yeast transcription factor GAL4 fusion system was used because GAL4 fusion proteins have little background interference in mammalian cells because of their origin. In addition, the GAL4 DBD directs fusion proteins to the nucleus, alleviating the concern that deletion mutants might disrupt the natural nuclear localization signal. To determine the minimal region necessary for transactivation, a series of RFLAT-1 carboxyl-terminal deletions fused in-frame to GAL4 DBD (AA 1–147) was created (Fig. 2*A*). These fusion plasmids were cotransfected into NIH 3T3 cells with a reporter construct containing five GAL4-binding sites upstream of the firefly luciferase gene. The GAL4-RFLAT-1 (full-length) fusion had little effect on activating the reporter gene. In some experiments, it appeared to repress transcription (Fig. 2*B*). Further deletions toward the amino terminus resulted in a gradual increase in reporter gene expression, with GAL4-RFLAT-1 1–35 giving 5–10-fold induction compared with GAL4 DBD alone (Fig. 2*A*). This result demonstrates that RFLAT-1/KLF13 AA 1–35 contains a transactivation domain that is able to enhance GAL4 transcription. In addition, a repression domain is contained within the carboxyl-terminal region of AA 1–168. The presence of the repression domain appears to inhibit the activation domain (AA 1–35) in the fusion system.

To further characterize the repression domain, a series of amino-terminal deletions of RFLAT-1/KLF13 fused to GAL4 DBD was generated (Fig. 2*B*). Among them, AA 67–168 has the strongest inhibitory effect with greater than 15-fold repression of transcription. AA 74–112 and 112–168 showed significant suppression, but the activity of neither was comparable with that of AA 67–168, suggesting that all of the sequences within AA 67–168 are required to reach maximum repression. In comparison, neither AA 168–250 nor AA 250–288 affected transactivation, demonstrating that neither the DNA-binding domain nor the carboxyl-terminal tail contains any activating or repressive activity. These data suggest that AA 67–112 contains a strong repression domain.

To ensure that these results were not due to differences in protein expression of the constructs, NIH 3T3 cells were transfected with each of the constructs, and protein extracts were prepared and analyzed by Western blot with an anti-GAL4 antibody. As shown in Fig. 2*C*, the appropriate size fusion proteins were produced from each construct, and their expression levels were comparable.

Deletion of the Activation Domain Impairs RFLAT-1/KLF13 as a Transcription Factor—To independently confirm that distinct transcriptional activation and repression domains exist within RFLAT-1, several RFLAT-1 truncated proteins were created in which either the amino-terminal activation domain or the middle repression domain was deleted but the potential nuclear localization signal and DNA-binding domain (AA 147–249) remained intact (Fig. 3*A*). The expression and nuclear localization of these protein products was demonstrated by transfection of the constructs into NIH 3T3 cells, followed by nuclear extract preparation and Western blotting using a polyclonal anti-RFLAT-1 antibody (Fig. 3*B*). These truncated proteins were then coexpressed together with a luciferase reporter gene driven by the *RANTES* promoter in Jurkat T-cells to test their ability to activate *RANTES* transcription. Compared with the full-length RFLAT-1, proteins with the amino terminus deleted (RFLAT-1 36–288 and RFLAT-1 67–288) completely lost their ability to induce the *RANTES* promoter (Fig. 3*A*). This observation further indicates that AA 1–35 contains the transcriptional activation domain. In contrast, truncation of the middle portion including the repression domain (RFLAT-1 1–35 + 147–288) increased target gene transactivation (Fig. 3*A*), suggesting that this domain mediates activity in suppressing transcription. In comparison, removal of the very end of the carboxyl tail (RFLAT-1 1–249) had little effect on RFLAT-1 activity.

The DNA binding of RFLAT-1 truncated proteins to their native target *RANTES* promoter A/B site was examined by an EMSA using recombinant proteins. Full-length or truncated His tag RFLAT-1 proteins were produced in *Escherichia coli*, purified by Ni^+ columns, and incubated with a radiolabeled A/B oligonucleotide. As shown in Fig. 3*C*, the four truncated proteins all bound to the A/B site, consistent with the fact that they all contain DNA-binding domains similar to that of the wild type. More than one band was observed with RFLAT-1 1–35 + 147–288. The slower migrating species may represent dimers/oligomers because when using glutaraldehyde fixation followed by Western blot, dimer/oligomer species were detected in the protein preparation (data not shown). The specificity of DNA-bound bands was confirmed by cold competitions (data not shown). These results demonstrate that deletion of AA 1–35 or 1–67 does not affect RFLAT-1 DNA binding. Thus, the loss of transcriptional activity of the two mutants is most likely due to the removal of the intrinsic activation domain.

Negatively Charged Amino Acid Residues Are Important for the RFLAT-1 Transactivation Domain—The RFLAT-1/KLF13 activation domain (AA 1–35) does not share any characteristics with other well defined transcriptional activation motifs, such as a high percentage of acidic residues (Asp and Glu), glutamines (Gln), or prolines (Pro) (1). Instead, it is rich in hydrophobic (Ala and Val) residues and exhibits high homology with the amino termini of BTEB1 and BTEB4 (Fig. 1*B*). This region of BTEB1 contains one of its activation domains (AA 13–26) (25). We noted that RFLAT-1 AA 1–35 also contains several acidic and serine residues (Asp^8 and Glu^{13} and the Ser^{17}, Ser^{19}, and Ser^{20} cluster) embedded among the hydrophobic residues. These residues are also found in BTEB1 and BTEB4 (Fig. 1*B*). To further characterize the RFLAT-1 activation domain and to identify amino acid(s) critical for transactivation, site-directed mutagenesis on GAL4-RFLAT-1 1–35 was performed (Fig. 4). Mutations of D8A, E13A, S20A, and S17A/S19A/S20A substantially impaired the activity of GAL4-RFLAT-1 1–35 in transfection assays, suggesting that these residues are all important in mediating transcriptional activation. A mutant in which the negatively charged residues Glu^{32} and Ser^{33} that are missing in BTEB1 and BTEB4 were changed to alanines also showed significant reduction in activity (Fig. 4). Mutation of the hydrophobic valine at position 7 to alanine also decreased activity to basal level (data not shown), indicating that both hydrophobic and acidic residues are important to transactivation. Similar expression of GAL4-RFLAT-1–35 and its point mutation proteins was revealed by Western blot (data not shown), indicating that differences in their transcriptional activities are due to the mutations themselves.

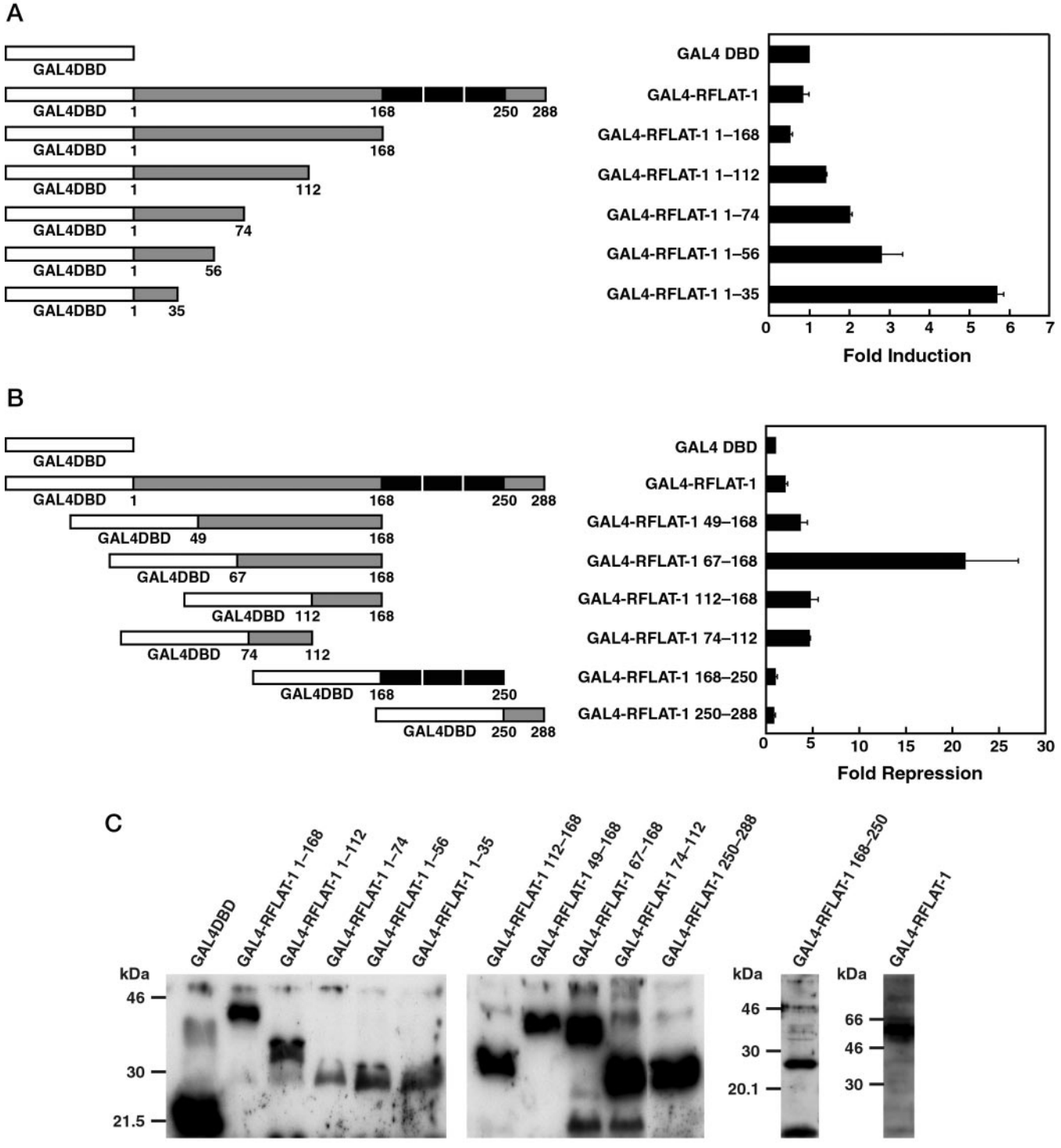


FIG. 2. **Identification of transcriptional activation and repression domains of RFLAT-1/KLF13 by the GAL4 fusion assay.** Full-length or fragments of RFLAT-1/KLF13 as indicated were fused in-frame with the GAL4 DBD. *A* and *B*, 1 μg of fusion construct and 1 μg of reporter gene were cotransfected into NIH 3T3 cells, and the luciferase activity was measured. The effect of the respective constructs on luciferase activity is indicated as fold induction or repression over that by the control vector containing only GAL4 DBD. The transfection was normalized according to *Renilla* luciferase activity as described under "Experimental Procedures." The data are presented as triplicate or duplicate transfections and represent at least five independent experiments. *C*, 20 μg of each fusion construct indicted in *A* and *B* was transfected into NIH 3T3 cells, and nuclear extracts were made 36 h post-transfection. 15 μg of each extract was separated by SDS-PAGE and Western blotted with a polyclonal anti-GAL4 (1–147) antibody.

RFLAT-1 Contains Two Potent, Independent Nuclear Localization Signals—RFLAT-1 is a nuclear protein (7). To identify functional sequences responsible for its nuclear localization, the full-sized and truncated RFLAT-1 fused in-frame to GFP were expressed in COS-7 cells, and cellular localization of the fusion products was monitored by autofluorescence. Fluorescence of GFP alone is shown in Fig. 5*A* and is present throughout the cell. In contrast, the majority of full-sized RFLAT-1-GFP accumulated in the nucleus (Fig. 5*B*), indicating that it contains a strong NLS(s). Sequence analysis revealed that RFLAT-1 AA 147–168 contains a bipartite NLS (26), consisting of two clusters of basic amino acids separated by a short non-

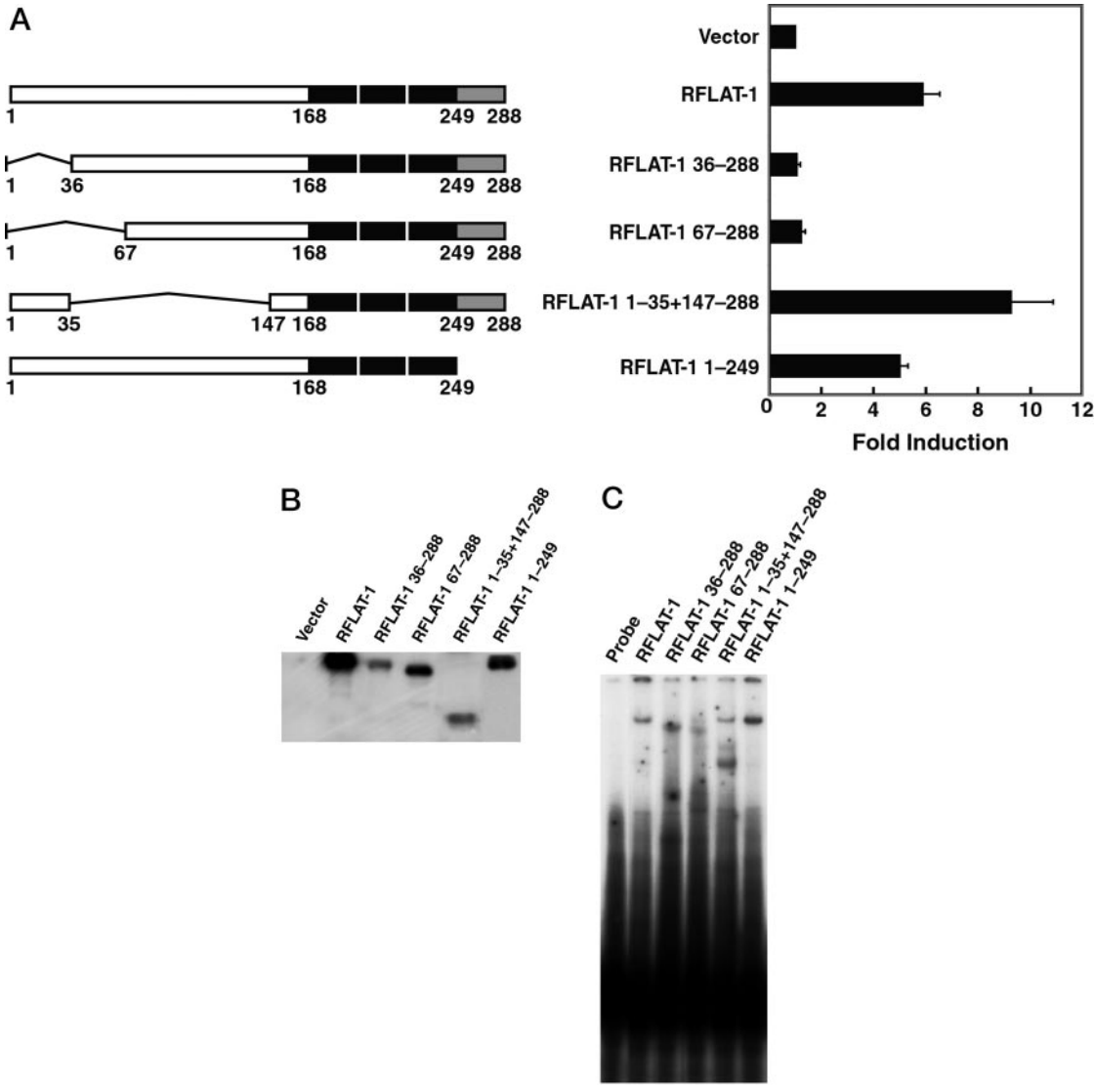


FIG. 3. **Transcriptional activation and repression domains of RFLAT-1/KLF13 are functionally separate and independent.** *A*, truncated RFLAT-1 proteins were made as described under "Experimental Procedures." 10 μg of each of the expression constructs was transfected into Jurkat T-cells with 10 μg of *RANTES* promoter-driven luciferase reporter gene and 0.1 μg of pRL-null plasmid. 48 h post-transfection, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are presented as fold induction over that by the control vector in triplicate transfections and represent five independent experiments. *B*, 1 μg of each of the construct expressing truncated RFLAT-1 proteins was transfected into NIH 3T3 cells, and nuclear extracts were made 48 h post-transfection. 10 μg of each extract was separated by SDS-PAGE and Western blotted with a polyclonal anti-RFLAT-1 antibody. *C*, recombinant RFLAT-1 wild type and mutant proteins were produced and purified from *E. coli* and tested for DNA binding. 1 μg of each recombinant protein was incubated with a radiolabeled probe containing the *RANTES* promoter A/B site. The resulting complexes were separated on a 8% nondenaturing acrylamide gel.

basic peptide. Thus, a fusion protein was constructed with AA 147–168 deleted (Fig. 5*C*). Surprisingly, this protein was still able to translocate into the nucleus, suggesting that in addition to AA 147–168, RFLAT-1 has other NLS. AA 147–168 is a potent NLS, because RFLAT-1 1–168-GFP accumulated in the nucleus (Fig. 5*D*), whereas RFLAT-1 1–146-GFP was spread throughout the cytoplasm (Fig. 5*E*). These results also confirm that AA 1–146 does not contain any NLS.

The effort to identify additional NLS was next focused on AA 169–288, the zinc finger DNA-binding domain plus the carboxyl tail. Fusion proteins RFLAT-1 169–288-GFP (Fig. 5*F*) and RFLAT-1 169–249-GFP (Fig. 5*G*) localized in the nucleus, whereas RFLAT-1 250–288-GFP did not (Fig. 5*I*), demonstrating that the second NLS is within AA 169–249, the zinc finger domain. The additional deletion of the third zinc finger (RFLAT-1 169–221) reduced nuclear accumulation significantly (Fig. 5*H*). These data demonstrate that RFLAT-1/KLF13 contains two potent, independent NLS; one is in the basic region immediately upstream of the zinc finger DNA-binding domain, and the other is within the zinc fingers. Each of the two signals is strong and sufficient to translocate GFP into the nucleus.

RFLAT-1/KLF13 Recognizes and Binds to the CTCCC Element—RFLAT-1/KLF13 binds to the A site of the *RANTES* promoter with high specificity. It does not bind to the B, C, or E regions, as demonstrated using partial recombinant RFLAT-1 protein (7). The A site (Fig. 6*A*) displays κB-like characteristics and was identified as a second NF-κB binding site by Moriuchi *et al.* (27). However, RFLAT-1 does not bind to an oligonucleotide containing a classical κB site derived from the immunoglobin promoter (7), demonstrating that the RFLAT-1 consensus sequence differs from the κB site. In EMSA, no complexes were observed using nuclear extracts of late activated T-cells with an oligonucleotide in which the four cytosine residues on the 3′ end of the A site were missing, suggesting that these residues are most critical for recognition

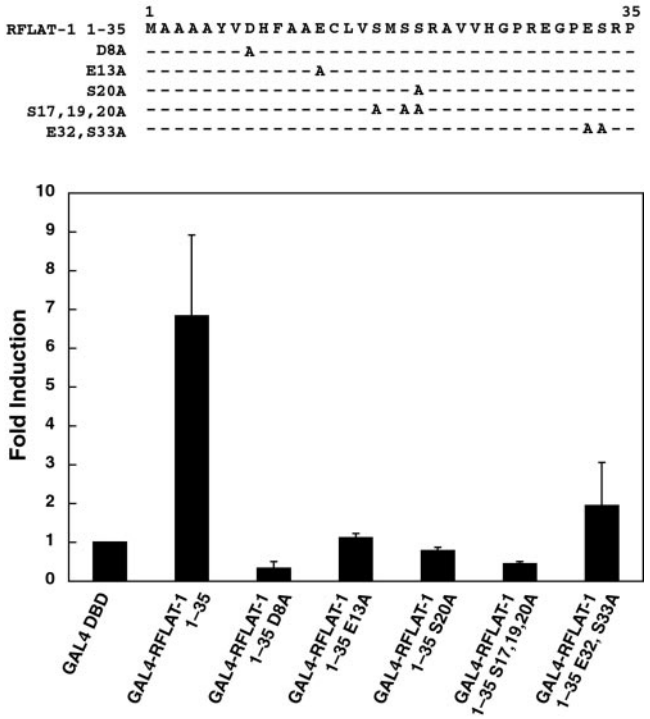


FIG. 4. **Identification of critical amino acids for RFLAT-1/KLF13 transactivation domain.** Selected amino acids as indicated were mutated to alanines within RFLAT-1 AA 1–35 in the GAL4 fusion protein. The resultant mutant constructs were cotransfected with the GAL4 reporter gene into NIH 3T3 cells, and the activity of each mutant is presented as fold induction over that of the construct containing only the GAL4 DBD.

and binding (28). Because all KLFs recognize related GC-type elements and CACCC boxes (4), we predicted that the central sequence for RFLAT-1/KLF13 recognition on the A site is likely to be the 3′ part of CTCCC. To test this, a series of mutated oligonucleotides were radiolabeled and used in EMSAs with full-length recombinant RFLAT-1/KLF13 protein (Fig. 6*A*). Mutation of the 3′ two cytosine residues (CTCCCC → CTCCTT) reduced binding to RFLAT-1. Mutation of all four cytosines (CTCCCC → CTTTTT) abolished protein binding, demonstrating that these cytosine residues are critical for RFLAT-1 recognition. In addition, mutation of the 5′ single cytosine residue (CTCCCC → TTCCCC) decreased binding significantly. In comparison, mutation of the thymidine (CTCCCC → CCCCCC) and sequences 5′ to the CTCCC box (AAA→GGG and GG→AA) reduced but did not eliminate binding, suggesting that they play minor roles in recognition. Destruction of the CTCCC box by reversing the sequence to TCTTT completely abrogated binding to recombinant RFLAT-1 (Fig. 6*A*).

The RFLAT-1 binding site on the *RANTES* promoter is similar to a number of previously identified DNA sequences that interact with known KLFs. It has also been reported that RFLAT-1 binds to the BTE consensus sequence and the CACCC box (9, 21). To determine the affinity of RFLAT-1 to published KLF DNA binding sequences, cold competition experiments were performed. As shown in Fig. 6*B*, EKLF (*lanes 9–11*) and BTE (*lanes 15–17*) oligonucleotides competed for binding slightly better than the wild type RFLAT-1 sequences. GKLF (*lanes 12–14*) competed as efficiently as the wild type. In comparison, a synthetic oligonucleotide containing the binding site for Sp1 (*lanes 6–8*) competed relatively poorly for binding. Because the binding sites for BTE, EKLF, and GKLF are all GT-like, the Sp1-binding site is GC-rich; this result suggests that RFLAT-1 may preferably recognize and bind to GT boxes.

The Intact CTCCC Box Is Necessary and Required for RFLAT-1-mediated RANTES Promoter Transcription—A subset of mutations described above were then introduced into the *RANTES* promoter by PCR-based site-directed mutagenesis. Both wild type and mutant promoters were transfected into Jurkat T-cells together with a *RFLAT-1* expression construct. Compared with the wild type promoter with an intact RFLAT-1 binding site, the A-2 mutation (CTCCCC → CTCCTT, see Fig. 6*A*) reduced but did not eliminate induction of *RANTES* expression by RFLAT-1 (Fig. 7*A*). In comparison, both the A-4 (CTCCCC → CTTTTT) and A-9 (CTCCC → TCTTT) Fig. 6*A* mutations resulted in complete loss of RFLAT-1-mediated *RANTES* promoter activation (Fig. 7*A*). These results are consistent with the EMSA data and suggest that an intact CTCCC box sequence is absolutely required for RFLAT-1 to activate the RANTES gene.

In T-cells, RFLAT-1 and NF-κB protein (p65 and p50) are both expressed late (3–5 days) after activation, and they drive RANTES gene expression in a synergistic fashion (7). On the *RANTES* promoter, both RFLAT-1 and NF-κB binding sites are between the CAAT and TATA boxes, with the NF-κB site downstream and adjacent to the RFLAT-1 site. To examine whether an intact RFLAT-1 binding site is necessary for synergy, the wild type *RANTES* promoter and the A-9 mutation were compared in cotransfection experiments. Introduction of all three proteins (RFLAT-1, p65, and p50) resulted in a significant synergistic induction of the *RANTES* promoter. This synergy is completely blocked by the disruption of the CTCCC box (Fig. 7*B*), suggesting that this sequence on the promoter is required for synergy.

Taken together, these data demonstrate that the RFLAT-1/KLF13 recognition and binding sequence on the *RANTES* promoter is the CTCCC box. RFLAT-1/KLF13 is also able to bind equally well to the other consensus GT boxes, and to a less degree, the GC boxes. The intact DNA binding sequence is required for RFLAT-1-mediated *RANTES* induction. In addition, it is required for the synergy observed between RFLAT-1 and NF-κB proteins. Although we do not know whether protein-protein interaction is involved between the two transcription factors, it is certain that the DNA-protein interaction between RFLAT-1 and CTCCC box is absolutely required for achieving synergy.

DISCUSSION

RFLAT-1/KLF13 was first identified as part of a search for late expressed T-cell transcription factors regulating *RANTES* transcription in T lymphocytes (7). In the present study to determine the structure-function relationship of RFLAT-1, a transcriptional activation domain, a repression domain, and two nuclear localization signals have been identified. Combined with information from other reports (21), our current model of the RFLAT-1 structural/functional domains is summarized in Fig. 8. RFLAT-1/KLF13 belongs to the recently identified and expanding KLF family. The evolutionary phylogenetic tree of 19 members of this family shows that RFLAT-1/KLF13, BTEB1/KLF9, and BTEB4/DRRF are most closely related and may form a subfamily. BTEB1/KLF9 was first cloned based on its ability to bind to the BTE, a GC-rich sequence present in the promoter of the cytochrome P-450IA1 (CYP1A1) gene (29). It functions as an activator for multiple GC box-containing promoters such as the SV40 early promoter but acts as a repressor of single GC box containing promoters like that of the CYP1A1 gene. The transcriptional activation domain of BTEB1 was mapped to two regions at the amino terminus: AA 13–26 and AA 58–68 (25). BTEB4 was recently cloned from human pancreas (30), but little information is available as to its function and domain structure. It may be an ortholog of the mouse DRRF, which is important for modulat-

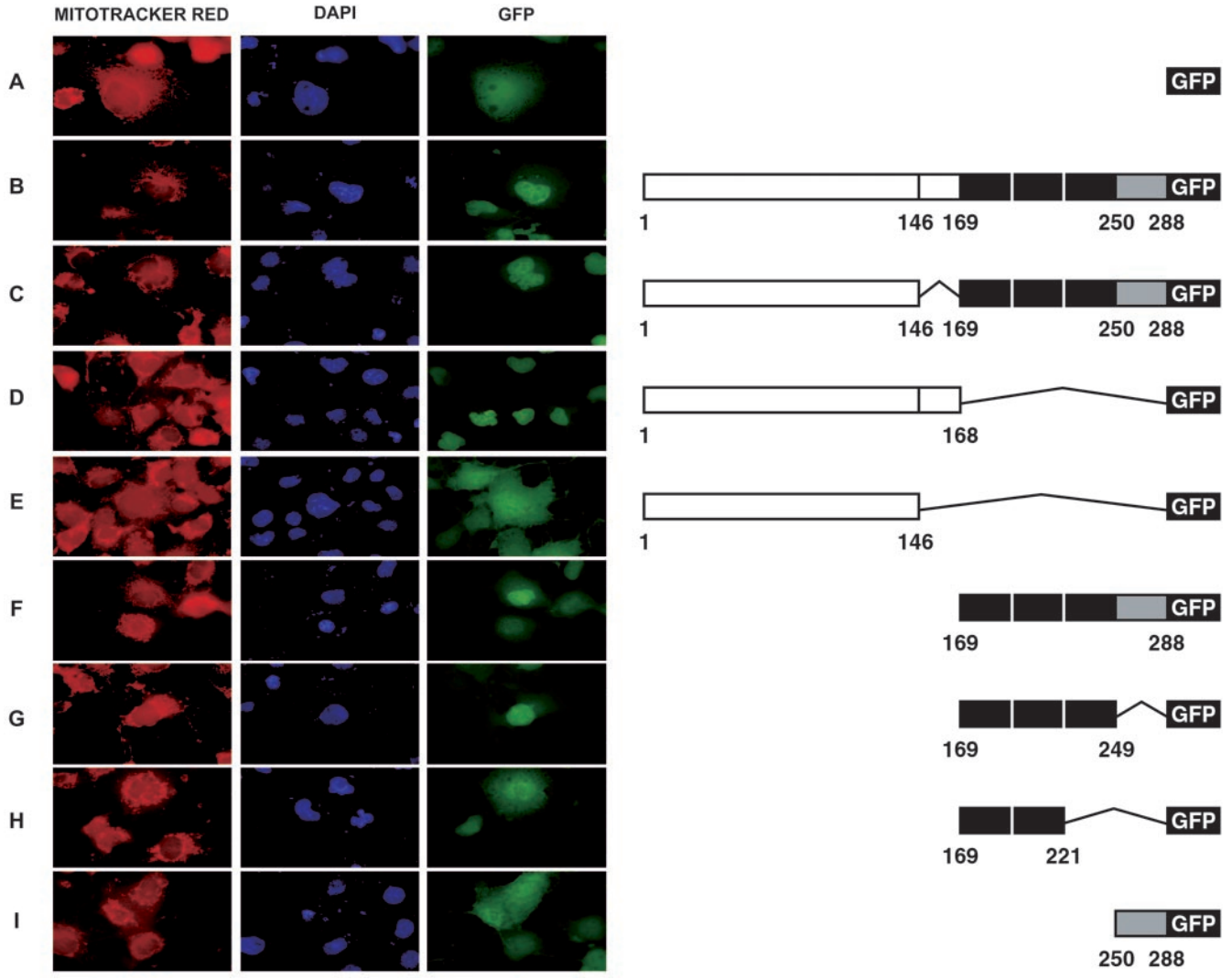


FIG. 5. **Cellular localization of GFP fusion proteins.** The various RFLAT-1-GFP fusion constructs used in the experiments are depicted on the *right side*. Fluorescence microscopy for COS-7 cells transfected by each construct is shown in *panels A–I*. The green color shows GFP autofluorescence (*GFP*). The whole cell and the nucleus are visualized by MitoTracker Red (*MITOTRACKER RED*) and DAPI autofluorescence (*DAPI*), respectively.

ing dopaminergic transmission in the brain (31). In addition to the similar DNA-binding motifs shared by all family members, these three proteins also share homology within their transactivation domains. As shown in Fig. 1*B*, the amino termini of these three factors are very similar. This region contains one of the activation domains of BTEB1/KLF9 (25) and is the activation domain of RFLAT-1/KLF13 identified in this study. It will be interesting to determine whether this region also mediates transactivation by BTEB4.

The Transcriptional Activation Domain of RFLAT-1/KLF13—The activation domains of RFLAT-1/KLF13 and BTEB1/KLF9 differ from the well characterized activation domains of many transcription factors, including other KLFs. For example, the transactivation domain of Sp1 is glutamine-rich (10), that of BTEB2 is proline-rich (32), and those of EKLF and GKLF are acidic-rich (13, 16). The activation domains of RFLAT-1/KLF13 and BTEB1/KLF9 have a high content of hydrophobic residues, with a small number of acidic and serine residues embedded within them (Fig. 2*B*). Mutagenesis studies on RFLAT-1 indicate that the highly conserved acidic residues and their neighboring hydrophobic residues are equally important in mediating transactivation. Thus, they may represent a new module responsible for transcriptional activation. It is generally accepted that the role of activation domains is to mediate interaction between sequence-specific transcription factors and basal transcriptional machinery (33). For example, the acidic residues may contact the target by charged interactions, and glutamine residues are involved in hydrogen bonding. In the case of RFLAT-1 and BTEB1, we predict that the contact between their transactivation domains with the basal transcriptional apparatus may involve both charged residues and hydrophobic interaction. The interaction may be initiated by contact between charged amino acids and reinforced and stabilized by stronger hydrophobic forces. Although it is currently not known whether the general components of the initiation complex or RNA polymerase II itself is the target for RFLAT-1, BTEB1, and BTEB4, it seems reasonable to predict that they probably all interact with the same protein or a similar region.

The Repression Domain of RFLAT-1/KLF13—Although many KLFs function only as activators or repressors, some KLFs are bifunctional, containing both activation and repression domains. Most of the repression domains have been identified using the GAL4 fusion system, like those of EKLF (13), GKLF (15), and LKLF (17). RFLAT-1/KLF13 is an activator for the RANTES gene, but, when fused with the GAL4 DBD, it acts as a repressor (Fig. 2*B*). This observation provides the first evidence that RFLAT-1/KLF13 may be a bifunctional tran-

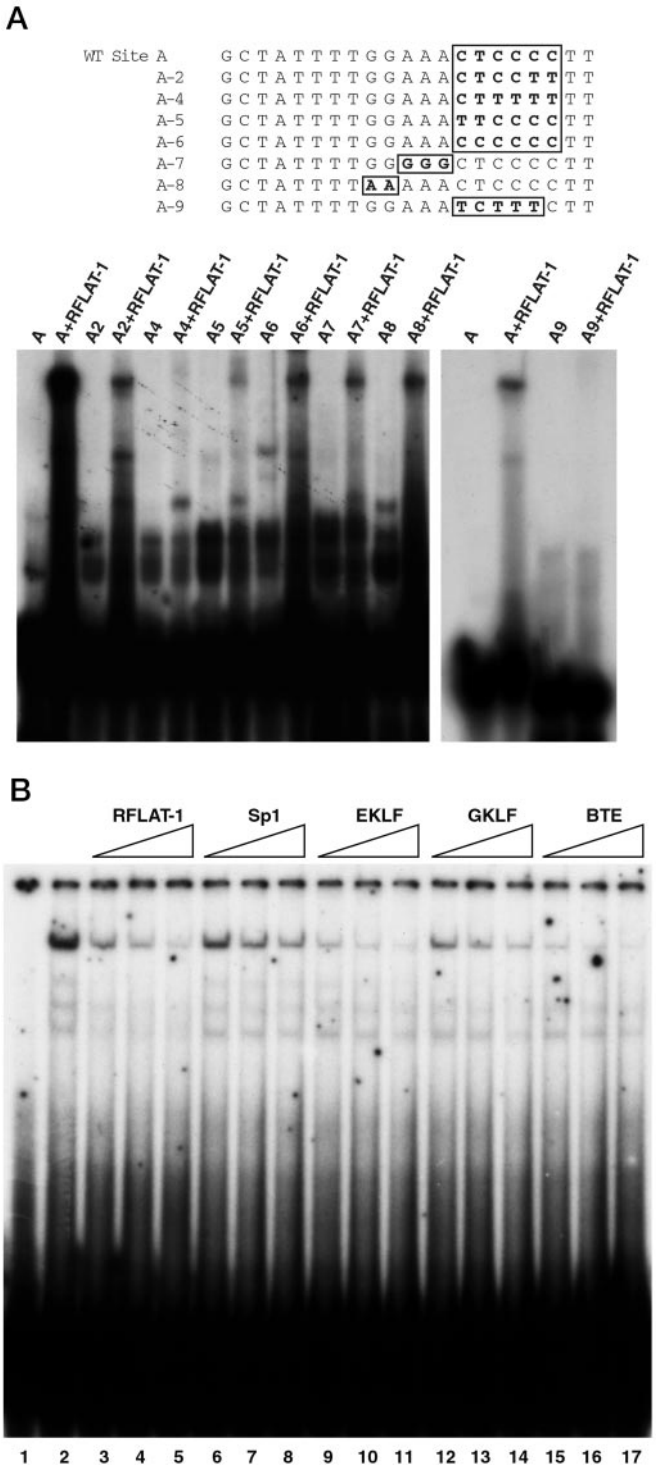


FIG. 6. **Critical nucleotide residues for RFLAT-1/KLF13 recognition and binding.** *A*, the indicated mutations were introduced into the oligonucleotide derived from the human *RANTES* promoter A site. These oligonucleotides were radiolabeled and mixed with 4 μg of full-length RFLAT-1/KLF13 recombinant protein produced from *E. coli* in an EMSA assay. *B*, competition experiments using established transcription factor binding sites. Radiolabeled A/B oligonucleotide and 3 μg of recombinant RFLAT-1 protein were used in each EMSA. *Lane 1*, free probe; *lanes 2–17*, probe plus RFLAT-1 protein. With the exception of *lane 2*, all of the lanes contained unlabeled competitor oligonucleotides. For each cold competitor, increasing amounts of competitor DNA in the order of 5-, 10-, and 20-fold molar excess over the probe were added to the reaction. The minimal essential binding sequence for corresponding KLFs is in *bold type*.

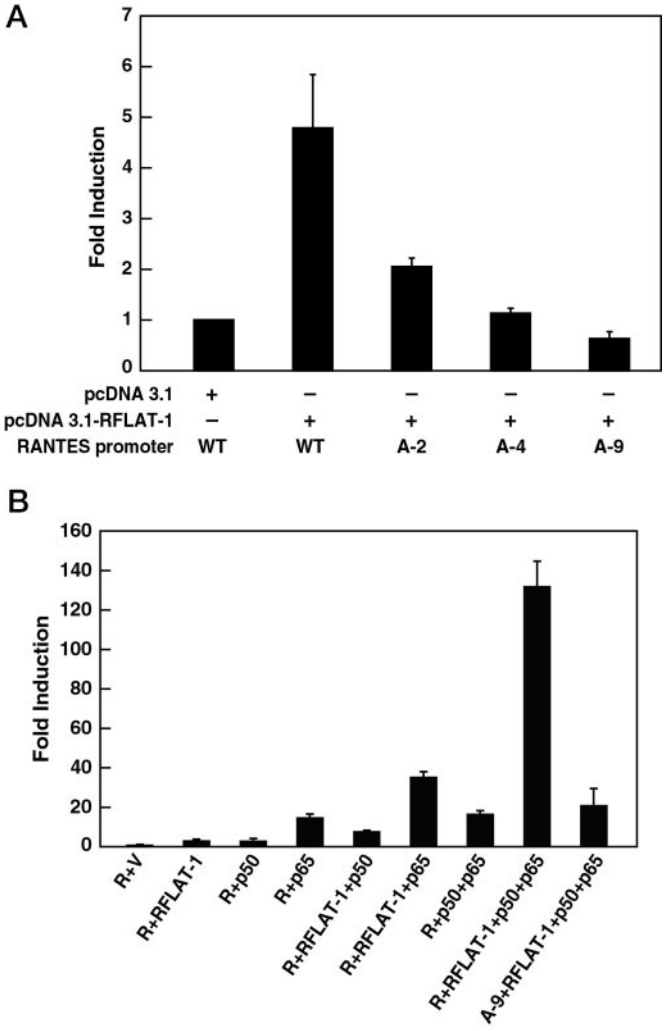


FIG. 7. **CACCC box is required for RFLAT-1-mediated *RANTES* transcription.** *A*, the A-2, A-4, or A-9 mutations were introduced into the *RANTES* promoter (−195) by PCR-based mutagenesis. The wild type (*WT*) and mutant reporter genes (10 μg) were cotransfected into Jurkat T-cells with either empty vector (pcDNA3.1, 10 μg) or RFLAT-1 expressing construct (pcDNA3.1-RFLAT-1, 10 μg) in the presence of pRL-null (0.1 μg). 48 h post-transfection, the firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. The data are presented as fold induction with activation of the empty vector set at 1. The data are presented as triplicate transfections and represent three independent experiments. *B*, cotransfection of RFLAT-1 and NF-κB genes in Jurkat T-cells. Jurkat cells were transfected with 10 μg of either wild type (*R*), or A-9 *RANTES* promoter-luciferase reporter genes, together with 10 μg of pcDNA3.1-*RFLAT-1*, 10 μg of pcDNA3.1-*p50*, 10 μg of pcDNA3.1-*p65*, or 10 μg of each of the three plasmids in different combinations. For each transfection, the total amount of plasmid used was equal and was achieved by using the empty vector pcDNA3.1 for compensation. The data are presented as fold induction over that of the empty vector, representing triplicate transfections and three independent experiments.

scription factor. Deletion analysis revealed that the minimum region conferring maximum repression is AA 67–168. This region shows no obvious sequence similarity with any other proteins, including its closest family members, BTEB1 and BTEB4 (Fig. 1*B*), but it is rich in alanines (19%) and prolines (20%), which are common residues in a few of the known

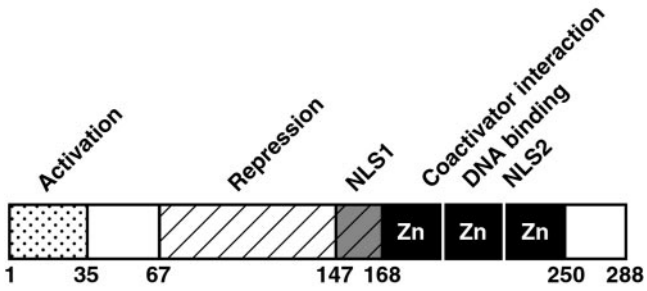


FIG. 8. **Functional domains of RFLAT-1.** The RFLAT-1 minimum activation domain is localized to AA 1–35, whereas the repression domain resides in AA 67–168. There are two NLS; one is the basic region upstream of the DNA-binding domain (AA 147–168), and one is the zinc finger DNA-binding domain. The zinc finger domain has also been shown to mediate interaction with coactivators such as CBP/p300 and p300/CBP-associated factor (21).

repression domains (34). These residues are also known to mediate protein-protein interactions. Although no repression domain has been identified for BTEB1, this factor was first cloned as a repressor for the rat *CYP1A1* promoter (29). Additionally, the mouse BTEB4/DRRF can act as either an activator or repressor on dopamine receptor promoters (31). These data suggest that, in addition to the well known bifunctional EKLF subfamily (EKLF, LKLF, and GKLF), the RFLAT-1/KLF13 subfamily of KLFs can also be activators and repressors depending upon the targeting promoters, the interacting proteins, and the cellular contacts.

In general, the mechanisms of transcriptional repression are less well understood than those mediating activation. It has been proposed that there are three mechanisms for repression: interference with (*a*) activator DNA binding; (*b*) the activity of DNA-bound activators; or (*c*) the general transcription machinery (34). It has long been known that some KLFs are transcriptional repressors, but only recent reports describe the mechanisms of suppressive activities of selective KLFs. BKLF confers its repressive activity by binding to the cellular protein CtBP2 (carboxyl-terminal binding protein 2) (35). EKLF interacts with the corepressors mSIN3A and histone deacetylase 1 through its zinc finger domain and suppresses transcription (14). The inhibitory domain of LKLF binds to a cofactor WWP1, an E3 ubiquitin ligase, which results in attenuated transactivation (17). In the above cases, the mechanisms of repression are all at the intermolecular level and involve interaction with corepressors. In the GAL4 system, fusion of the RFLAT-1/KLF13 repression domain with GAL4 DBD did not change its DNA binding affinity (data not shown). On the contrary, the presence of the repression domain completely masked or quenched the activity of the activation domain, suggesting that its mode of action is either by covering the activation surface or interacting and interfering with the basal transcriptional machinery. In the RFLAT-1, *RANTES* promoter system, deletion of the repression domain resulted in increased RFLAT-1 transactivation activity, which further indicates the inhibitory function of this domain.

The NLS of RFLAT-1—Most eukaryotic transcription factors contain one or more NLS that can be recognized by nuclear transport proteins. These proteins translocate the transcription factors across the nuclear membrane in an ATP-dependent fashion (36). Two types of NLS have been defined: the "core" NLS, which contains four or more arginine and lysine residues within a hexapeptide that is frequently flanked by acidic residues or prolines and glycines, and the "bipartite" NLS, which consists of two clusters of basic amino acids separated by a short nonbasic peptide (26). Based on sequence search, a bipartite NLS was identified within RFLAT-1 AA 147–168. This is a potent NLS because it is sufficient to direct GFP into the nucleus. However, it is not the only NLS on RFLAT-1 because deletion of this signal did not affect the nuclear transport. Moreover, this bipartite NLS sequence is not conserved on BTEB1 and BTEB4 (Fig. 1*B*), and little is known about the NLS of the two BTEB proteins.

Although no putative NLS (core or bipartite) is found within the finger region, the second NLS of RFLAT-1 was localized to the three zinc fingers of the DNA-binding domain. Deletion of the last zinc finger significantly reduced nuclear transport, perhaps indicating that all three fingers are required for optimal nuclear translocation. The zinc fingers of other KLFs, such as those of GKLF (26), UKLF (37), and EKLF (38), have also been shown to act as strong NLS. In some cases, a "global" structure of zinc fingers, rather than specific sequences, serves as an NLS (39, 40). In others, basic residues within the zinc fingers are critical determinants for nuclear localization (38). Thus, we conclude that for KLFs, the zinc finger domain may have multiple functions. This domain of RFLAT-1/KLF13 is involved in nuclear localization, interaction with coactivators CBP/p300 and p300/CBP-associated factor, and binding to DNA.

RFLAT-1 Recognition Sequences—All KLFs recognize GC-rich regions, but their precise recognition sequences are slightly different. Sp1 binds to GC-rich box GGGGCGGGG, whereas EKLF recognizes GT-rich CACCC sequence. Our mutation analyses demonstrated that RFLAT-1/KLF13 recognizes and binds to the CTCCC box of the *RANTES* promoter. Competition studies showed that it also binds to other previously published GT- or GC-rich sequences. However, its affinity to the GT box is somewhat stronger than that to the GC box. This intact CTCCC sequence is required for RFLAT-1-mediated RANTES gene transcription in T-cells. The importance of this sequence for the synergistic effect between RFLAT-1 and NF-κB proteins supports the enhanceosome model in which both activators and their cognate DNA fragments are all required to exert optimal gene transcription (7).

In conclusion, the functions of selected structural regions of RFLAT-1/KLF13, a member of the Krüppel-like transcription factor family, were analyzed. Distinct transcriptional activation and repression domains and two potent, independent nuclear localization signals were identified. Unique structural features may help define the mechanisms of action of RFLAT-1/KLF13 in regulating gene expression. Additionally, this information provides new insights into functional similarity and differences among the large and growing Krüppel-like transcription factor family.

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Functional Domains and DNA-binding Sequences of RFLAT-1/KLF13, a Krüppel-like Transcription Factor of Activated T Lymphocytes

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