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RFLAT-1: A New Zinc Finger Transcription Factor that Activates RANTES Gene Expression in T Lymphocytes

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Summary

RANTES (*R*egulated upon *A*ctivation, *N*ormal *T* cell *E*xpressed and *S*ecreted) is a chemoattractant cytokine (chemokine) important in the generation of inflammatory infiltrate and human immunodeficiency virus entry into immune cells. *RANTES* is expressed late (3–5 days) after activation in T lymphocytes. Using expression cloning, we identified the first "late" T lymphocyte associated transcription factor and named it "*R*ANTES *F*actor of *L*ate *A*ctivated *T L*ymphocytes-1" (RFLAT-1). RFLAT-1 is a novel, phosphorylated, zinc finger transcription factor that is expressed in T cells 3 days after activation, coincident with *RANTES* expression. While Rel proteins play the dominant role in RANTES gene expression in fibroblasts, RFLAT-1 is a strong transactivator for *RANTES* in T cells.

Introduction

RANTES (*R*egulated upon *A*ctivation, *N*ormal *T* cell *E*xpressed and *S*ecreted) is a member of the large and growing family of immunoregulatory cytokines called chemokines. The functions of chemokines include attracting blood leukocytes to sites of inflammation (Baggiolini et al., 1997; Schluger and Rom, 1997), regulating leukocyte maturation, trafficking and homing, and the development of lymphoid tissues (for recent review, see Nelson and Krensky, 1998; Baggiolini, 1998). RANTES belongs to the C-C chemokine subfamily. It is a potent chemotactic agent for monocytes, T lymphocytes (Schall et al., 1990), eosinophils (Kameyoshi et al., 1992; Rot et al., 1992), basophils (Dahinden et al., 1994), and natural killer cells (Maghazachi et al., 1994; Taub et al., 1995). It also causes degranulation of basophils, respiratory burst in eosinophils, and activation of T cells (Kuna et al., 1992; Alam et al., 1993; Bacon et al., 1995). Thus, RANTES appears to play an important role in both acute and chronic phases of inflammation.

RANTES and the closely related chemokines, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, may also play a role in resistance to human immunodeficiency virus (HIV) infection. Cocchi and colleagues showed that RANTES, MIP-1α, and MIP-1β inhibit infection of HIV in $CD8^+$ T cells in vitro and that these chemokines are highly expressed in some patients who are HIV^+ but do not progress to AIDS (Cocchi et al., 1995). Subsequently, several groups showed that the C-C chemokine receptor CC-CKR5, which selectively binds to these chemokines, is a coreceptor for HIV entry into target cells (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). Based on these observations, RANTES and its receptors have been evaluated as potential therapeutic targets for immune-related diseases and AIDS (Nelson and Krensky, 1998). Thus, understanding the molecular basis for control of RANTES gene expression, especially in T cells, may help in the design and development of novel immunotherapies for a variety of diseases.

The RANTES gene was originally identified as a cDNA during a general screen for genes expressed at a "late" stage of T cell activation (Schall et al., 1988). When peripheral T cells are triggered through the T cell receptor, they enter a 3–7 day maturation process that involves commitment, proliferation, and terminal differentiation (Crabtree, 1989; Ullman et al., 1990). Commitment occurs rapidly after the binding of the T cell receptor with the specific antigen and is accompanied by immediate induction of a number of genes such as protooncogenes and interleukins. Once T cells are activated, they synthesize new DNA and proliferate. At 3–5 days after activation, T cells enter the phase of terminal differentiation, characterized by the expression of functional genes and the generation of effector cells such as cytotoxic T lymphocytes or helper T cells. Nearly 100 genes have been shown to be induced "early" upon T cell activation. Studies on one of these, interleukin-2, led to the identification of a transcription factor NFAT (nuclear factor of activated T lymphocytes), which mediates T cell activation (Shaw et al., 1988; Crabtree and Clipstone, 1994). The elucidation of the calcineurin-NFAT signaling pathway provided insight into the mechanism of action of the immunosuppressive drugs cyclosporin and FK506 (Schreiber and Crabtree, 1992). Compared to the number of genes identified as early genes, fewer genes are newly expressed at the T cell differentiation stage, and the molecular basis of late expression is poorly understood. Unlike other members of the chemokine family, which are expressed early (Ullman et al., 1990), *RANTES* mRNA is induced late (3–5 days) after T cell activation. Together with the genes encoding perforin, granzymes A and B, and granulysin, it is expressed at high levels in terminally differentiated T cell lines (Ortiz et al., 1997). This timing appears unique to T cells, as many other cell types, including fibroblasts, epithelial cells, and monocytes/macrophages express *RANTES* within hours after stimulation (Ortiz et al., 1995). Studies on the *RANTES* promoter and its *cis*-regulatory elements implicate the involvement of late T cell transcription factors in this unique expression pattern (Nelson et al., 1996; Ortiz et al., 1996). Work from our group led to the hypothesis that there is a "switch" of nuclear factors in maturing T cells (Ortiz et al., 1997). However, these late T cell transcription factors remain to be identified.

Here, we report the first cloning and characterization of a late transcription factor, which we have designated RFLAT-1 (*R*ANTES *F*actor of *L*ate *A*ctivated *T L*ymphocytes-1). RFLAT-1 is a novel protein belonging to the

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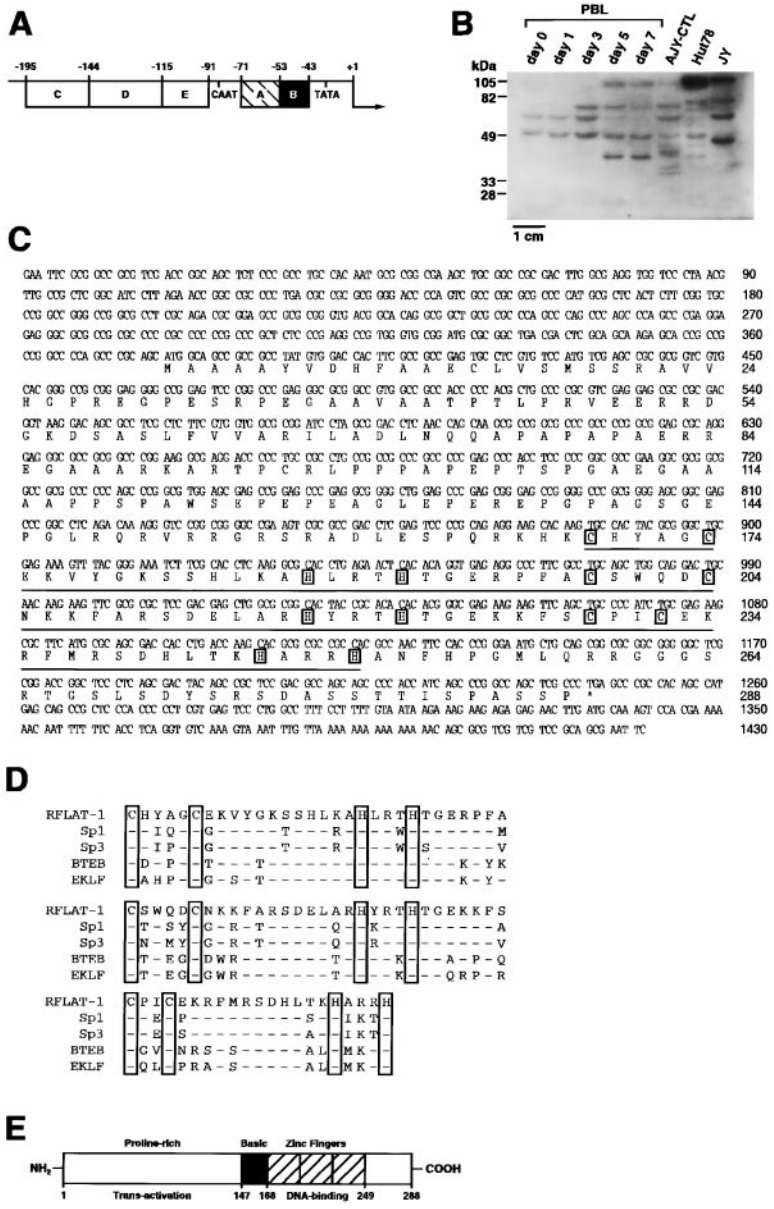

Figure 1. Southwestern Analysis, Nucleotide and Deduced Amino Acid Sequence of RFLAT-1, and Zinc Finger Domain Comparison of RFLAT-1 with Other Proteins

(A) Diagram of regulatory regions in the −195 *RANTES* promoter and their positions relative to transcription start site +1.

(B) 50 μg each of nuclear extracts from resting PBL (day 0), PHA activated PBL at days 1–7, AJY-CTL, mature T tumor line Hut78, and B lymphoblastoid JY cells were extracted and separated by 10% SDS-PAGE. Nuclear proteins were transferred onto a PVDF membrane and hybridized with [γ^{32}P] labeled A/B oligonucleotide. The membrane was washed and an autoradiogram was made by exposure to Amersham hyperfilm at −80°C for 3 days.

(C) Nucleotide and deduced amino acid sequence of RFLAT-1. The three zinc fingers are underlined and the highly conserved cysteine and histidine residues are shown in boxes.

(D) Comparison of the zinc finger domain of RFLAT-1 to those of other proteins. Similar amino acids are depicted by dashed lines. The two cysteine and two histidine residues for each zinc finger are boxed.

(E) Schematic diagram of the RFLAT-1 protein structure. The proline-rich putative transactivation domain, the three zinc fingers, and the short basic region are represented by an open box, three shadowed boxes, and a solid box, respectively. The numbers indicate the first and last amino acid of each domain.

TFIIIA-like zinc finger protein superfamily. RFLAT-1 specifically binds to the A site of the *RANTES* promoter (Figure 1A). It is a component of protein complexes binding to the A/B site that are induced 3 days after T cell activation. The RFLAT-1 protein itself is expressed in T cells 3 days after activation. Reporter gene assays demonstrate that RFLAT-1 is a strong activator of the *RANTES* promoter in T cells. In contrast, Rel proteins play the dominant role in RANTES gene expression in fibroblasts.

Results

Non-Rel Proteins Bind to the A/B Site of the *RANTES* Promoter

The *RANTES* promoter has been subdivided into five regions (A-E) based upon deletion studies and reporter gene assays (Figure 1A) (Nelson et al., 1993, 1996; Ortiz et al., 1996). The A site is very important for the promoter activity since point mutations or nonsense nucleotide replacement decreases the activity by 90% (Nelson et al., 1996; Moriuchi et al., 1997). The A site binds both Rel and non-Rel proteins, with the non-Rel protein being upregulated in T cell nuclear extracts on day 3 after activation, coincident with *RANTES* message induction (Nelson et al., 1996). The C site and E site also bind nuclear proteins that are strongly induced between days 3 and 5 after activation of normal peripheral blood T cells (Ortiz et al., 1996).

In order to identify the non-Rel protein binding to the A site, Southwestern blotting was performed. Nuclear extracts from resting peripheral blood lymphocytes (PBL), Phytohemagglutinin (PHA) P activated PBL on different days, and other cell lines were separated by SDS-PAGE and blotted on PVDF membranes. The membranes were hybridized with a radiolabeled oligonucleotide corresponding to the A/B region of the *RANTES* promoter and several nuclear proteins were detected

(Figure 1B). Proteins migrating at about 38 kDa, 75 kDa, and 105 kDa were detected in PBL only at late activation stages (days 3–7), showing kinetics similar to the factors detected by electrophoretic mobility shift assays (EMSA) (Nelson et al., 1996). When an oligonucleotide containing three tandem A sites (A3) was used as the probe, the 38 kDa protein showed an even higher binding affinity (data not shown).

Identification and Characterization of RFLAT-1

The results from the Southwestern analysis indicated that expression cloning could be used to identify proteins that bind to the A region of the *RANTES* promoter. A λgt11 cDNA library was constructed using poly(A)+ RNA from PBL on day 5 after activation by PHA, and the library was probed with radiolabeled A3 oligonucleotides. Upon screening, a cDNA clone (880 bp) was identified that contained three contiguous zinc fingers defined as a classical DNA binding motif (Mitchell and Tjian, 1989). This clone was used to screen the original library to obtain a longer cDNA (1430 bp), which contains an open reading frame of 864 bp defined by a potential ATG start codon (nucleotide 379–381), a TGA stop codon (nucleotide 1243–1245), and is flanked by 378 (5′) and 185 (3′) untranslated nucleotide sequences (Figure 1C). The first methionine is surrounded by a consensus Kozak sequence (GCCCGCAGC***ATG***G). A stop codon TGA is found 43 bp upstream in-frame with the start codon. This open reading frame encodes a 288 amino acid polypeptide with a calculated molecular mass of 31,680 daltons. A search of the GenBank database with the BLAST algorithm failed to identify any similar protein or cDNA sequence. Based on its function and expression, we have named it RFLAT-1 (for *R*ANTES *F*actor of *L*ate *A*ctivated *T L*ymphocytes-1).

The deduced amino acid sequence contains three contiguous TFIIIA-like zinc-finger motifs at the C terminus. This domain shows 73%, 72%, 65%, and 65% sequence similarity to those of Sp1 (Kadonaga et al., 1987), Sp3 (Kingsley and Winoto, 1992), EKLF (Bieker, 1996), and BTEB (Sogawa et al., 1993) proteins, respectively (Figure 1D). Adjacent to the N terminus of the zinc finger motif is a short sequence rich in basic amino acids, which does not show any obvious sequence homology to the consensus sequence for the basic domain of either Sp1 family members or helix-loop-helix-leucine zipper proteins (Kadonaga et al., 1987; Fisher et al., 1991). Outside of these domains, the RFLAT-1 protein (1–145) is notably rich in proline (24/145), serine (10/145), and alanine (30/145) residues, which are known to constitute activation domains for a number of transcription factors (Figure 1E) (Mitchell and Tjian, 1989).

To confirm that the 1430 bp cDNA clone encodes the full-length RFLAT-1 protein, the *RFLAT-1* cDNA was subcloned into the pcDNA3.1 vector and subjected to in vitro transcription and translation. The reaction produced a doublet protein band with an apparent molecular mass of 38 kDa that was not present when the control vector was used (Figure 2A), confirming the accuracy of the *RFLAT-1* sequence. Alkaline phosphatase treatment converted the slower migrating band to the faster migrating form (Figure 2A), indicating that the two bands are different forms of the protein, and that RFLAT-1 is

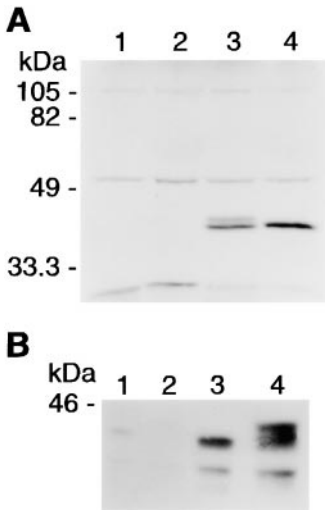

Figure 2. Characterization of the RFLAT-1 Protein

(A) *RFLAT-1* cDNA was subcloned into the pcDNA3.1 vector and subjected to in vitro transcription and translation in the presence of [^{35}S]-Met. Aliquots of the translated products from pcDNA3.1 (lane 1) or pcDNA3.1-*RFLAT-1* (lane 3) were separated by 10% SDS-PAGE. Other aliquots were treated with alkaline phosphatase for 1 hr at 37°C before loading onto the gel (lane 2, pcDNA3.1 + phosphatase; lane 4, pcDNA3.1-*RFLAT-1* + phosphatase). The gel was fixed, dried, and exposed for autoradiography.

(B) COS-7 cells were transfected with pcDNA3.1 (lane 2) or pcDNA3.1-*RFLAT-1* (lane 4). Thirty-six hours after transfection, cells were harvested and nuclear extracts were isolated. Aliquots of nuclear extracts were treated with alkaline phosphatase for 1 hr at 37°C (lane 1, pcDNA3.1 + phosphatase; lane 3, pcDNA3.1-*RFLAT-1* + phosphatase). Each sample representing equal number of cells was resolved by 10% SDS-PAGE and immunoblotted with anti-RFLAT-1.

likely to be phosphorylated. To further confirm this, COS-7 cells were transfected with pcDNA3.1-*RFLAT-1* and analyzed by Western blot for RFLAT-1 expression using polyclonal antibodies generated against recombinant RFLAT-1. Bands at approximately 38 kDa and lower were detected only in *RFLAT-1* transfected cells, but not in empty vector transfected cells (Figure 2B). Alkaline phosphatase treatment converted some, but not all, of the slower migrating species into the faster migrating form (Figure 2B). These results confirm that the *RFLAT-1* cDNA clone contains the entire coding region of the protein and also indicate that RFLAT-1 is phosphorylated.

Tissue and Cellular Distribution of RFLAT-1

Northern blot analysis was performed using poly(A)+ RNA from 16 different adult human tissues. Two distinct transcripts (5 and 7.5 kb) were evident in all of the tissues analyzed, with the greatest abundance in PBL and thymus (Figure 3A). *RFLAT-1* expression was also assayed with poly(A)+ RNA from human cell lines of hematopoietic origin and found to be widely expressed (Figure 3B).

The intracellular localization of the RFLAT-1 protein was analyzed by immunofluorescence in COS-7 cells transiently transfected with *RFLAT-1* cDNA. The overexpressed RFLAT-1 protein was primarily localized to the nuclear compartment of transfected COS-7 cells (Figure 4B). RFLAT-1 staining was blocked specifically by addition of recombinant His_6-RFLAT-1 (data not shown). When

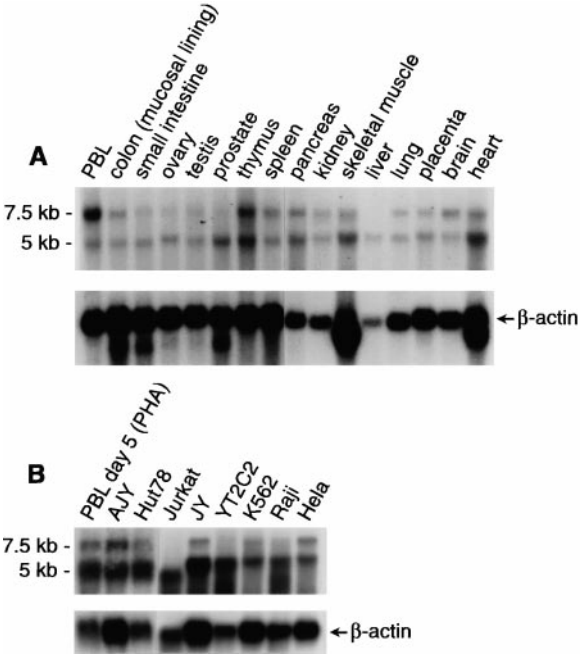

Figure 3. Tissue Distribution of RFLAT-1

(A) Northern blot analysis of poly(A)+ RNA from adult human tissues. A prepared multi-tissue membrane (Clontech) was hybridized with [α^{32}P] randomly labeled *RFLAT-1* cDNA probe. The same membrane was stripped and subsequently hybridized with a human β-actin probe to normalize for the amount of RNA.

(B) Northern blot analysis of 2 μg poly(A)+ RNA from different cell lines: PBL (day 5, PHA activated), AJY (CTL), Hut78 (mature T cell tumor), Jurkat (T cell tumor), JY (B lymphoblastoid), YT2C2 (NK-like tumor), K562 (erythroleukemia), Raji (B lymphoma), and HeLa (fibroblastoid). The same membrane was also hybridized with a β-actin probe.

cells were simultaneously stained with anti-RFLAT-1 and propidium iodide, RFLAT-1 and stained DNA colocalized in the nucleus (Figures 4C and 4D). The combination of cellular fractionation and Western blotting also shows the primary localization of RFLAT-1 in the nucleus of activated T cells (data not shown). For Jurkat, RFLAT-1 is only detected in the nucleus (Figure 6B).

RFLAT-1 Binds DNA Sequences of the A Region of the *RANTES* Promoter

A carboxyl terminal portion of RFLAT-1, including the zinc finger DNA binding domain (nucleotides 550–1430), was isolated through expression cloning using an oligonucleotide probe containing three tandem A sites of the RANTES promoter. The same phage clones did not bind to the E or C site when a trimer E oligonucleotide or tetramer C oligonucleotide were used as probes (data not shown). To further demonstrate its DNA binding specificity, the C-terminal portion of RFLAT-1 (amino acids 59–288) was expressed as a His_6-tagged recombinant protein and used for EMSA (Figure 5A). Incubation of His_6-RFLAT-1 with the A/B and A probes, but not the B probe, revealed retarded protein bands. It is likely that binding of RFLAT-1 to the A site enhances cooperative binding of the protein to the B site, thereby accounting for the lower mobility band seen in the EMSA using the A/B probe but not the A probe. His_6-RFLAT-1 does not bind to the E or C probes in the assays, consistent with the results from the expression screening. Although the A/B site is very similar to the consensus sequence of the NF-κB binding site, and both the p50-p50 homodimer and p65-p50 heterodimer bind to the A/B region (Nelson et al., 1996; Moriuchi et al., 1997), His_6-RFLAT-1 did not bind to the κB oligonucleotide (Figure 5A), which contains the consensus NF-κB recognition sequence derived from the immunoglobin promoter (Baeuerle and Henkel, 1994).

To determine whether RFLAT-1 is present in the protein complex binding to the A/B region of the *RANTES* promoter that is induced upon T cell activation, nuclear extracts were isolated from resting and activated PBL for supershift assays. Two prominent bands were detected in the activated PBL nuclear extracts with the A/B probe; band 2 shows later kinetics than band 1 (Figure 5B). Addition of polyclonal anti-RFLAT-1 to the gel shift reaction mixture disrupted the complex formation for both band 1 and band 2 (Figure 5C), indicating that RFLAT-1 is indeed present in both complexes. Similar results were obtained when either the A or B probe was used (data not shown). The ability of the anti-RFLAT-1 polyclonal antiserum to disrupt the DNA-protein complex was not surprising since the antiserum was raised against the C-terminal portion of RFLAT-1 that contains the DNA-binding domain. Commercially available antibodies to other DNA binding proteins were also evaluated. Anti-p50, anti-p65, but not anti-c-rel, also supershifted both band 1 and band 2 (Figure 5C; data not shown), demonstrating that NF-κB is also present in both of the DNA-protein complexes. Miyamoto et al. (unpublished data) reported that Sp1 and Sp3 are components of the protein complex binding to the A/B site that is induced by interleukin-1β in astrocytes. We used the same antibodies to test whether Sp1 or Sp3 are present in T cell A/B-protein complexes. Neither anti-Sp1 nor anti-Sp3 supershifted or disrupted the A/B DNA-protein complex, suggesting that Sp1 and its close family members are not involved in the upregulation of *RANTES* expression in activated T lymphocytes.

Nuclear extracts from other cell lines were also tested for A site binding. Both RFLAT-1 mRNA and protein are expressed in Jurkat, Hut78, JY, and HeLa cells (Figures 3B and 6B). Nuclear extracts from Hut78, a "mature" T cell tumor line that constitutively expresses low levels of RANTES, showed a similar A site binding pattern to that of nuclear extracts from PBL activated for 7 days with PHA. In contrast, nuclear extracts from Jurkat, JY, and HeLa, which do not express RANTES, did not show this pattern of binding to the A site (Figure 5D).

RFLAT-1 Is Expressed Late after T Cell Activation

RFLAT-1 is a component of the protein complex binding to the A/B region of the *RANTES* promoter. This complex is induced late (3–5 days) after T cell activation. To investigate these kinetics, total RNA from resting and activated PBL was isolated and RNA blots were probed with the 880 bp *RFLAT-1* cDNA fragment (nucleotides 550–1430). Both transcripts (5 and 7.5 kb) were detected at comparable levels in resting and activated PBL (Figure 6A). Therefore, during T cell activation, the *RFLAT-1*

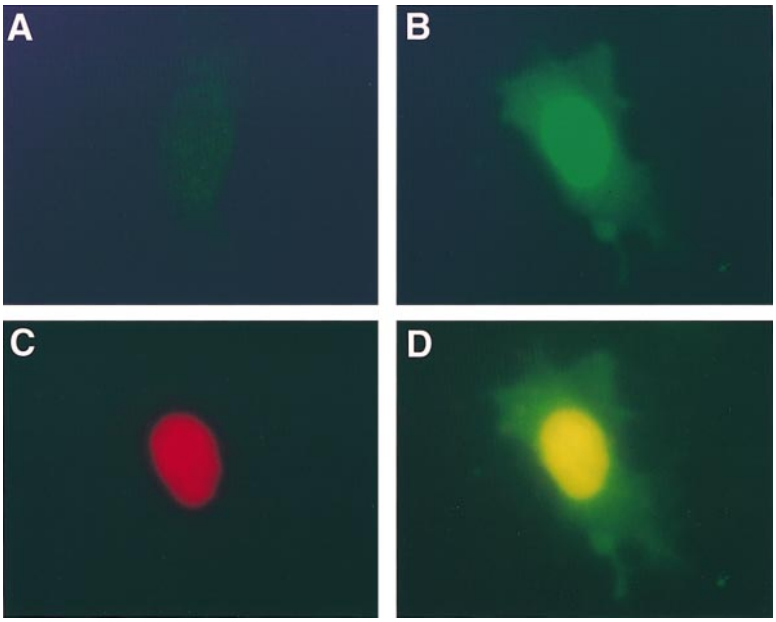

Figure 4. Cellular Localization of RFLAT-1

COS-7 cells were transfected with pcDNA3.1 or pcDNA3.1-*RFLAT-1*. After 36 hours, the transfected cells were fixed, permeabilized, labeled with antigen purified anti-RFLAT-1, and then stained with goat anti-rabbit FITC and anti-FITC Alexa 488. All the cells were simultaneously stained with propidium iodide to reveal the nucleus. (A) A COS-7 cell transfected with the pcDNA3.1 and stained with anti-RFLAT-1. (B) A COS-7 cell transfected with pcDNA3.1-*RFLAT-1* and stained with anti-RFLAT-1. (C) The same cell shown in (B) stained with propidium iodide. (D) Colocalization of RFLAT-1 (green) and stained DNA (red) in the nucleus (yellow).

steady-state mRNA level is constant. Nuclear extracts and cytoplasmic fractions were also isolated from quiescent and activated PBL for Western blot analysis. Proteins reactive with anti-RFLAT-1 antiserum migrating at 38 kDa and lower appeared in both PBL nuclear and cytoplasmic extracts only after day 3 of activation (Figure 6B), indicating that the RFLAT-1 protein is expressed at the late activation stage of T lymphocytes. Western analysis was also performed for the NF-κB subunits p50 and p65. Both p50 and p65 proteins were enriched in the nucleus of activated T cells between day 3–5 after activation. In the cytoplasmic fractions, the protein levels of p50 and its precursor p105 steadily increased following cellular activation (Figure 6C), but cytoplasmic p65 peaked at day 3 and then decreased. Conversely, nuclear p65 began to increase at day 5 (Figure 6D). These results show that the NF-κB protein is also increased at the late stage of T cell activation, and this increase is in part due to nuclear translocation.

RFLAT-1 Functions as a Transactivator for the RANTES Gene

To investigate the role of RFLAT-1 in the expression of *RANTES*, pcDNA3.1-*RFLAT-1* was introduced into Jurkat T cells together with luciferase reporter constructs derived from the *RANTES* promoter. Cotransfection of *RFLAT-1* with the *pGL2-R-luciferase* construct increased the promoter activity by 10-fold (Figure 7A), demonstrating that RFLAT-1 can function as a transactivator for the *RANTES* promoter. A similar degree of induction was observed when a reporter gene construct containing only three A/B sites was used for the cotransfection, indicating that RFLAT-1 recognizes the A/B sequences. This was confirmed with *pGL2-R-luciferase* constructs in which either the A site, the B site, or both sites were replaced with nonsense oligonucleotides. Replacement of the A site decreased the induction more than replacement of the B site, indicating that the A site is more important than the B site in the RFLAT-1 mediated effect. When the C or E sites were disrupted, RFLAT-1 mediated induction was also impaired, indicating that these sites also play roles in transactivation. However, this decrease is most likely not mediated through direct binding to RFLAT-1 (as shown in EMSA, Figure 5A).

To evaluate the role of RFLAT-1 in *RANTES* expression in non-T cells, *RFLAT-1* cDNA and reporter gene constructs were introduced into NIH3T3 fibroblasts. RFLAT-1 also transactivated the *RANTES* promoter in fibroblasts, but the fold induction was only about half of that achieved in Jurkat T cells (Figure 7B). In addition, only the A and B sites, but not the E and C sites, affected RFLAT-1-mediated *RANTES* induction in fibroblasts. This suggests that RFLAT-1 functions through different mechanisms in Jurkat T cells and in NIH3T3 fibroblasts.

To further investigate this possibility and to dissect the roles of RFLAT-1 and the NF-κB family members in RANTES gene induction in different cell types, the cDNA of the *p65* subunit and the *p50* subunit of the NF-κB protein were also subcloned into pcDNA3.1 and cotransfected into either Jurkat T cells or NIH3T3 fibroblasts for reporter gene assays (Figures 7C and 7D). In T cells, both RFLAT-1 and p65 induced RANTES gene expression, but RFLAT-1 was a much more potent transactivator than p65, as shown by the greater degree of induction (10-fold versus 3- to 4-fold) when the same amount of plasmid was introduced (data not shown). When *RFLAT-1*, *p65*, and *p50* were simultaneously overexpressed, a synergistic effect was observed (Figure 7C). Identical cotransfection assays were performed in NIH3T3 fibroblasts (Figure 7D). In this case, p65 played the dominant role in the transactivation of RANTES gene expression. p65 transactivation activity was so strong that it masked the effect of RFLAT-1 (40-fold induction by p65 alone versus 5-fold induction by RFLAT-1 alone) (Figure 7D). When all three cDNAs (*RFLAT-1*, *p65*, and *p50*) were overexpressed, RFLAT-1 enhanced the transcriptional induction by p65, but this effect was not

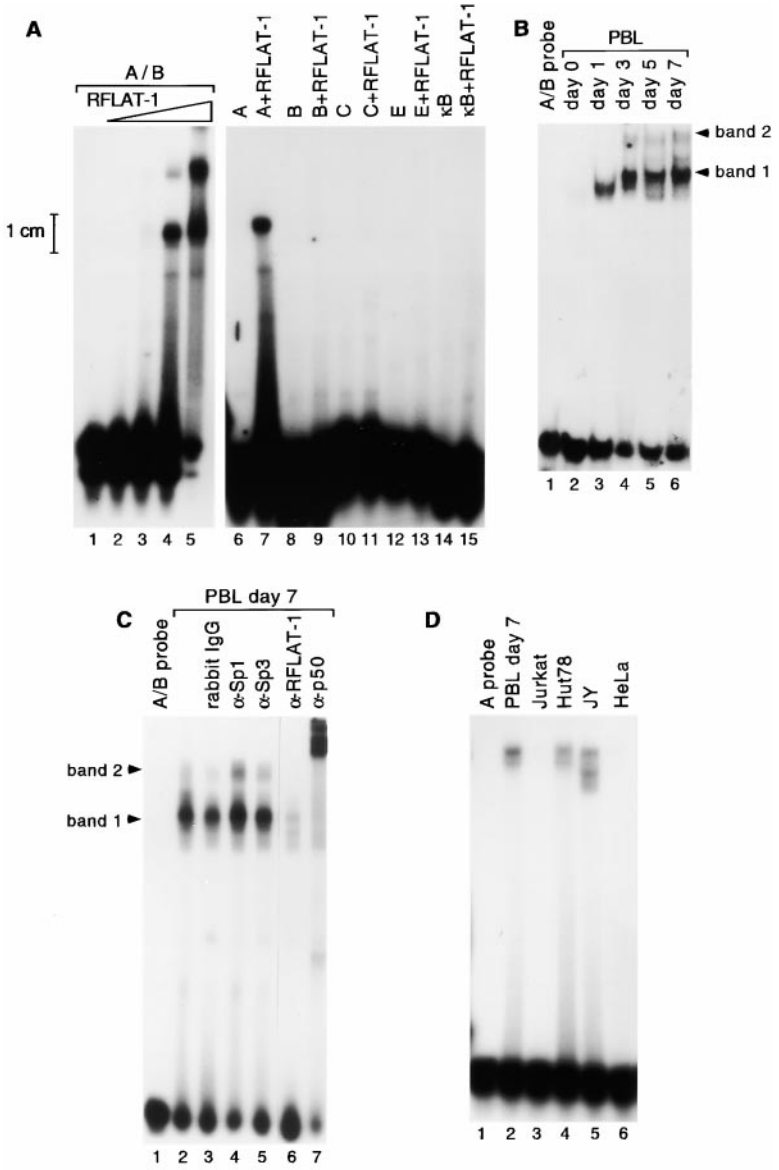

Figure 5. DNA Binding Activity of RFLAT-1

(A) The C-terminal 880 bp portion of the *RFLAT-1* cDNA was subcloned into the pET28a(+) vector to make a His_6-tagged fusion protein. The purified and refolded recombinant His_6-RFLAT-1 was used for EMSA with [γ^{32}P] end-labeled oligonucleotides as indicated. Lane 1–5: 0 μg, 1.25 μg, 2.5 μg, 5 μg, and 10 μg of His_6-RFLAT-1 was added to the A/B probe, respectively. 5 μg of His_6-RFLAT-1 was used for lanes 7, 9, 11, 13, and 15. The reaction mixtures were separated on a 8% nondenaturing polyacrylamide gel and exposed for autoradiography.

(B) 1 μg each of nuclear extracts from resting PBL (day 0), or PHA activated PBL (days 1–7) were used for EMSA with end-labeled A/B probe. Reaction mixtures were separated on a 8% nondenaturing polyacrylamide gel. Protein complexes binding to the A/B oligonucleotide are indicated by arrows.

(C) 1 μg of nuclear extract from PHA activated day 7 PBL was included in EMSA for lanes 2–7, and the A/B probe was used for lanes 1–7. 4 μg of individual antibodies were added to the assay as indicated. The reaction mixtures were separated on a 6% nondenaturing gel to resolve the supershifted complexes.

(D) 1 μg each of nuclear extract from PHA activated day 7 PBL, Jurkat, Hut78, JY, and HeLa cells, and A probe was used for EMSA.

synergistic. Thus, NF-κB is more potent than RFLAT-1 as a transactivator of RANTES gene expression in fibroblasts, while the reverse is true in T cells.

Discussion

RANTES is a chemoattractant molecule that plays a pivotal role in both acute and chronic inflammation and can block HIV infection in vitro. In normal T cells, expression of the RANTES gene is late, 3–5 days after activation. In the context of an immune response, late *RANTES* expression may be important in amplification and propagation of an inflammatory state (Ortiz et al., 1995). However, until recently, little was known about the molecular mechanisms underlying the induction of genes at this late stage of T cell activation (Ortiz et al., 1997). Characterization of the transcriptional regulation of *RANTES* in T cells will help us understand largely unexplored molecular mechanisms underlying gene expression.

RFLAT-1 Is a Developmentally Regulated Protein in T Lymphocytes

RFLAT-1 is the first late T cell transcription factor identified to date. However, as shown by Northern analysis, *RFLAT-1* is widely expressed. The ubiquitous expression of RFLAT-1 suggests that it may play a more general role in transcriptional regulation, and that additional mechanisms may further modulate its activity. For example, in cell lines that do not express RANTES, such as Jurkat, HeLa, and resting fibroblasts, RFLAT-1 protein is detected in the nucleus by Western blotting, but nuclear extracts do not contain material that binds to the *RANTES* A site (Figure 5D; data not shown). These results suggest that the regulation of RFLAT-1 activity, at least at the level of DNA binding, is complicated. Our results also indicate that during T cell activation, RFLAT-1 function is regulated mainly at the protein level. The steady-state level of the *RFLAT-1* message does not change during T cell maturation, indicating that *RFLAT-1* expression is

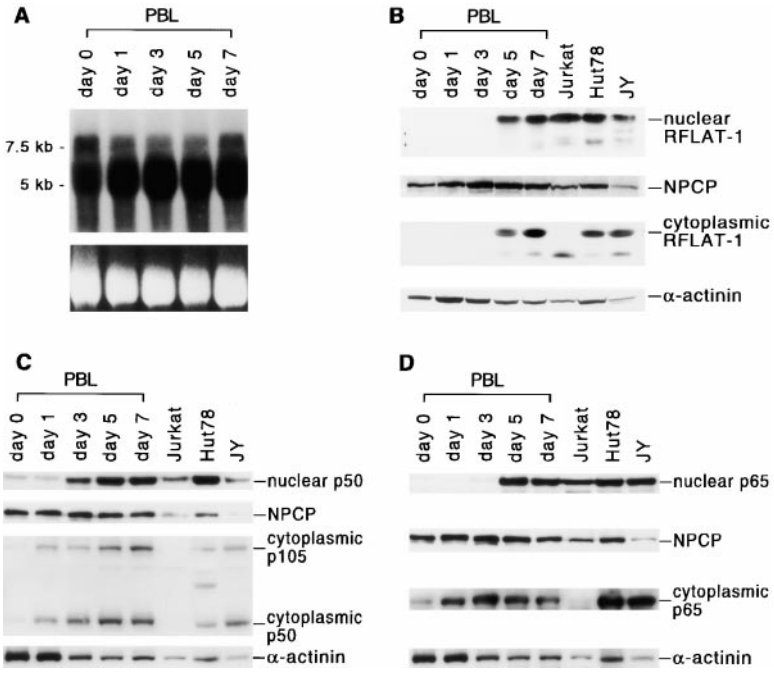

Figure 6. The RFLAT-1 Protein Is Induced Late in Activated T Cells

(A) Northern analysis of *RFLAT-1* expression in T cell activation. 20 μg of total RNA from resting PBL (day 0) and PHA activated PBL (days 1–7) were isolated and separated on a 1% formaldehyde-agarose gel and transferred onto a nylon membrane. The membrane was hybridized with [α^{32}P] randomly labeled *RFLAT-1* cDNA probe under stringent conditions. The 28s rRNA of each sample was visualized by ethidium bromide staining to normalize for the amount of RNA loaded.

(B) Western analysis of RFLAT-1 in nuclear and cytoplasmic extracts of activated T cells. Nuclear or cytoplasmic extracts from resting PBL (day 0), and PHA activated PBL (day 1–7), Jurkat, Hut78, and JY were isolated. 200 μg of each sample were subjected to SDS-PAGE and immunoblotted with antigen purified anti-RFLAT-1. As loading controls, the nuclear blot was reprobed with a monoclonal antibody against nuclear pore complex protein (NPCP); the cytoplasmic blot with a monoclonal anti-α-actinin.

(C) Western analysis of p50 in activated T cells. 50 μg of each of the samples described in (B) were prepared for Western blotting using anti-p50.

(D) Western analysis of p65 in activated T cells. Similar blots as described in (C) were probed with anti-p65.

not transcriptionally controlled. Nevertheless, Western analysis shows that the RFLAT-1 protein is detected in both nuclear and cytoplasmic fractions only 3 days after T cell activation and increases further by day 7, suggesting that RFLAT-1 protein synthesis is induced developmentally during T cell activation. Alternatively, the late appearance of RFLAT-1 may also be due to other posttranslational mechanisms.

RFLAT-1 Is a Member of the Zinc Finger Protein Superfamily

Zinc finger motifs have been identified in a number of DNA binding factors and other regulatory proteins (Mitchell and Tjian, 1989). Since RFLAT-1 contains three tandem TFIIIA-like zinc fingers located at the C terminus, it belongs to the Sp1-like superfamily. Unlike Sp1 and more closely related proteins, RFLAT-1 does not contain a highly conserved glutamine-rich transactivation domain (Courey et al., 1989). Instead, a putative transactivation domain rich in proline and alanine residues is present at the N terminus. While the overall structure of RFLAT-1 is similar to erythroid Krüppel-like factors (EKLF, BTEB, EZF), on the protein level, its putative transactivation domain does not show any obvious sequence similarity to them or other proline-rich domain containing proteins. Proline-rich domains may contain discrete activation and repression subdomains (Chen and Bieker, 1996; Yet et al., 1998). They can also mediate protein-protein interactions as shown for the association of the TFIIB component of the basal transcription machinery, and TBP-associated factors (TAF) with other transcriptional activators (Kim and Roeder, 1994; Chiang and Roeder, 1995). In the *RANTES* promoter, RFLAT-1 binds to the A site, while Rel proteins bind to the B and probably the A sites. The A/B site resides between the CCAAT and TATAAA boxes, bringing RFLAT-1 and Rel proteins in close proximity to the basal transcriptional machinery. However, whether RFLAT-1, Rel proteins, and basal transcription factors indeed physically interact with each other remains unclear.

Is *RANTES* Expression in T Cells Controlled by an Enhanceosome?

RANTES gene expression is regulated through distinct mechanisms in T cells and other cell types. In fibroblasts and epithelial cells, *RANTES* is induced immediately (within hours) by proinflammatory stimuli such as TNF-α and IL-1β. This rapid induction allows the immediate attraction of blood leukocytes to the site of inflammation and is most likely achieved by the binding of Rel proteins, and possibly other early response factors, to the promoter. Compared to non-T cells, the upregulation of *RANTES* expression in activated T cells is more complex and is developmentally controlled. First, a variety of transcription factors are involved, including both early factors (Rel proteins) and late regulated DNA binding proteins (RFLAT-1 and others). These factors may synergize to induce *RANTES* expression as shown for RFLAT-1 and Rel proteins. Second, as a transactivator, RFLAT-1 requires not only its own *cis*-acting element, but also intact adjacent, and even distal, DNA segments in order to exert its effect. Together, these findings support an enhanceosome model for *RANTES* gene transcription in T cells (Miyamoto et al., unpublished data).

An enhanceosome is a nuclear protein complex assembled at a given enhancer to confer a network of

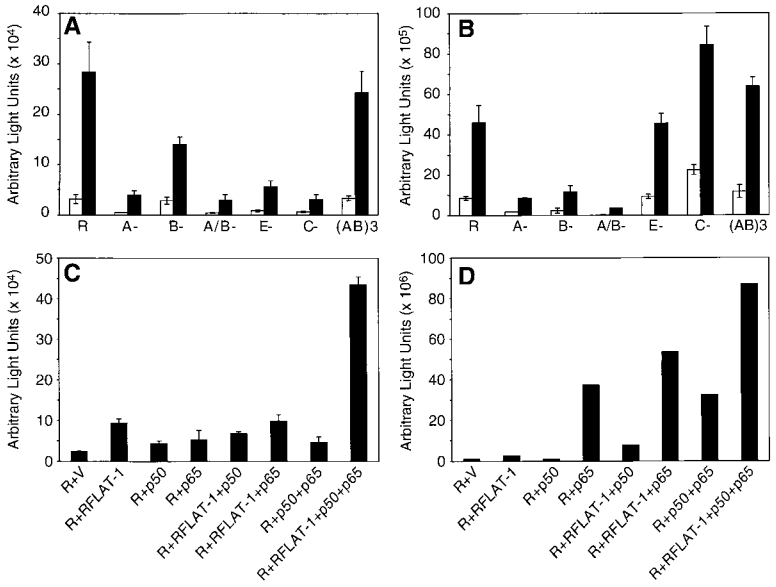

Figure 7. RFLAT-1 Is a Transactivator for the RANTES Gene

(A) RFLAT-1 transactivates the RANTES gene in Jurkat T cells. 10 μg of the pcDNA3.1 (open bars) or pcDNA3.1-*RFLAT-1* (solid bars) plasmids were electroporated into Jurkat cells together with 10 μg of luciferase reporter constructs derived from the *RANTES* promoter. R: pGL_2-*R-luciferase* containing the complete 200 bp *RANTES* promoter region immediately upstream of the transcription start site. A-, B-, A/B-, E-, and C-: reporter gene constructs derived from pGL_2-*R-luciferase* with either the A, B, A/B, E, or C site replaced by nonsense oligonucleotides. (AB)3: pGL_2-SV40-(AB)3-Luciferase. Thirty-six hours after electroporation, cells were harvested for luciferase assays.

(B) RFLAT-1 transactivates the RANTES gene in NIH3T3 fibroblasts. 5 μg of the pcDNA3.1 (open bars) or pcDNA3.1-*RFLAT-1* (solid bars) plasmids were introduced into NIH3T3 cells together with 5 μg of luciferase reporter gene constructs: R, A-, B-, A/B-, E-, C-, or (AB)3. Thirty-six hours after transfection, cells were harvested and luciferase activity was measured.

(C) Cotransfection of RFLAT-1 and Rel genes in Jurkat T cells. Jurkat cells were transfected with 10 μg of pGL_2-*R-luciferase*, 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 plasmid amount was equal (40 μg) and was achieved by using the empty vector pcDNA3.1 as compensation.

(D) Cotransfection of RFLAT-1 and Rel genes in NIH3T3 fibroblasts. Similar cotransfections as described in Figure 7C were performed in NIH3T3 cells, except 5 μg of each plasmid was used, and the total plasmid amount for each transfection was 20 μg. Data in figure 7 is presented as the mean ± SD of triplicate transfections or the mean of duplicate transfections and represent at least three independent experiments.

protein-protein and protein-DNA interactions. The interactions of the various components of an enhanceosome allow cooperativity and transcriptional synergy (Carey, 1998). The enhanceosome model for the RANTES gene best explains the findings to date, including its unique and precise expression pattern in T cells. In addition to gene activators, DNA-bending proteins such as HMG(I/Y) also participate in the enhanceosome structure, probably by binding to the E site (Song et al., unpublished data). Further characterization of the proposed *RANTES* enhanceosome will require identification of other late proteins that bind to the C and E sites.

Rel proteins are known activators for a wide range of genes. They also play important roles for *RANTES* expression, as shown by our group and others (Nelson et al., 1996; Moriuchi et al., 1997). The interaction and synergy of Rel proteins with other proteins is clear. NF-κB and NFAT interact to coordinately activate IFN-γ gene transcription (Sica et al., 1997). NF-κB and STAT1 are thought to mediate the synergistic effect of TNF-α and IFN-γ by cooperatively activating the expression of IRF-1 (Ohmori et al., 1997). In T cell activation, NF-κB is well documented as an immediate early gene (within 30 min) that activates early genes such as interleukin-2 (Crabtree, 1989; Ullman et al., 1990), but its expression and role in late activation stages of T cells has not been previously examined. In this report, we investigated the kinetics of the NF-κB protein in a later time frame and found that both the p50 and p65 subunits are upregulated late during cellular activation. The coexistence of RFLAT-1 and NF-κB provides supporting evidence for their synergistic effect on the *RANTES* promoter in T cells, but whether they physically interact still remains to be determined.

Conclusions

We have identified and characterized a novel transcription factor that regulates *RANTES* expression in T lymphocytes. This is the first late T cell transactivator described. RFLAT-1 and its *cis*-acting binding sequence provide a potentially interesting new target for immunotherapy. Downregulating *RANTES* expression may prove useful as a therapy for a variety of autoimmune and other inflammatory diseases, while upregulating *RANTES* expression may prove therapeutic for AIDS or cancer.

Experimental Procedures

Nuclear Extract Preparation and Southwestern Blotting

Human PBL were isolated over Ficoll-Hypaque (ICN Biomedicals, Inc., Costa Mesa, CA). Cells (2×10^6/ml) were stimulated with 5 μg/ml PHA (DIFCO, Detroit, MI) in RPMI Medium 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% FCS (HyClone, Logan, UT)/2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA) for up to 7 days. Nuclear and cytosolic extracts were prepared as described (Durand et al., 1988), with minor modifications (Ortiz et al., 1996). The protein concentration of the extracts was determined by Bradford assay using the Bio-Rad protein assay reagent. 50 μg of each nuclear extract was separated by 10% SDS-PAGE and the proteins were transferred onto a PVDF membrane. The membrane was then subjected to a denaturation-renaturation procedure by incubation in binding buffer (10 mM Tris-Cl [pH 7.5], 80 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1% glycerol) supplemented with decreasing amounts of guanidine (6 M, 4.5 M, 2.25 M, 1.13 M, 0.56 M, 0.28 M, 0.14 M, and 0 M) at 0°C. The membrane was blocked with 5% non-fat milk in binding buffer, hybridized with ^{32}P end-labeled oligonucleotide

(0.1–0.2 pmole/ml in 0.25% non-fat milk in binding buffer) at 25°C for 5 hr, washed, and exposed to Amersham Hyperfilm.

Library Construction and Screening

PBL were cultured with PHA-P for 5 days and total RNA was extracted (Chomczynski and Sacchi, 1987). Poly(A)+ RNA was further isolated using a QIAGEN kit (QIAGEN Inc., Santa Clarita, CA). 5 μg of poly(A)+ RNA was used to construct a λgt11 library using the SuperScript Choice System (Life Technologies, Gaithersburg, MD). The recombinant λ phage were packaged with GigapackIII Gold Packaging Extract (Stratagene, La Jolla, CA) and amplified. For expression cloning, λ phage plaques were induced with IPTG at 37°C for 6 hr and transferred to PVDF membranes. The denaturation-renaturation treatment and hybridization protocol was the same as that for Southwestern blotting except that hybridization was only for 1 hr at 25°C, and the binding buffer was supplemented with 10 μg/ml of denatured, sonicated calf thymus DNA. A total of 6×10^6 (3× library complexity) plaques were screened with a ^{32}P end-radiolabeled A3 oligonucleotide. The oligonucleotide sequence was GGGAATTCGAATTCGCTATTTTGGAAACTCCCCTTAGGGCTATTT TGGAAACTCCCCTTAGGGCTATTTTGGAAACTCCCCTTAGGCTC GAGCTCGAGGG. Positive phage clones were isolated and phage DNA was purified with a Wizard Lambda Preps DNA Purification System (Promega, Madison, WI). The cDNAs were excised from the λgt11 vector by restriction digestion and subcloned into a plasmid vector for sequencing. The same library was used for DNA-DNA screening following the protocols described by Ausubel et al., 1989. 2.4×10^6 plaques were screened with a ^{32}P randomly labeled 880 bp cDNA fragment from *RFLAT-1* to obtain the 1430 bp complete sequence.

Northern Blotting

Total RNA and poly(A)+ RNA was isolated as described above. Multi-human tissue blots with poly(A)+ RNA were purchased from Clontech (Palo Alto, CA). Using Clontech ExpressHyb Hybridization solution, Northern blots were probed with the *RFLAT-1* cDNA fragment or human β-*actin* cDNA under high-stringency conditions according to the manufacturer's instructions.

Transient Transfections and Luciferase Reporter Gene Assays

A series of *RANTES* promoter pGL2-luciferase reporter constructs were described previously (Nelson et al., 1993; Ortiz et al., 1996). *RFLAT-1* cDNA (EcoRI fragment) was excised from the λgt11 vector and subcloned into the CMV-promoter-driven mammalian expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). *p65* cDNA (HindIII-BamHI fragment) and *p50* cDNA (HindIII–XbaI fragment) were excised from pBluescript vector (gifts from G. Nolan, Stanford University) and subcloned into pcDNA 3.1. COS-7 and NIH3T3 fibroblasts were transiently transfected by the calcium phosphate method (Sambrook et al., 1989). Jurkat T cells were transiently transfected by electroporation as described previously (Nelson et al., 1993). 36 hr after transfection, the cells were harvested and luciferase activity was determined using a Luciferase Assay System Kit (Promega, Madison, WI) following the manufacturer's instructions. Luciferase activity was measured over 30 sec in a Wallac/EG&G Lumat LB 9507 Luminometer. Transfection efficiency was normalized by protein content using Bradford protein assays. In some transfections, CMV-*lacZ* plasmid was cotransfected to determine transfection efficiency and β-Galactosidase assays were performed according to the instructions accompanying the reporter lysis buffer reagent.

Western Blotting and In Vitro Transcription/Translation Assay

The 880 bp fragment of the *RFLAT-1* cDNA was fused into the pET-28a(+) vector to generate the expression vector. The pET-*RFLAT-1* plasmid was transformed into bacterial strain BL21(DE3)plysS and protein expression was induced by Isopropyl-β-D-thiogalactoside (IPTG). Bacterial pellets were dissolved in 6 M guanidine-HCl/0.05 M Tris-HCl and lysed by sonication. Recombinant His_6-RFLAT-1 was purified using a Ni^+ column and refolded in 5× volume of buffer containing 0.75 M Arginine, 0.05 M Tris-HCl (pH 8.0), 0.05 M KCl, and 0.0001 M EDTA. Proteins were then dialysed against PBS and concentrated. His_6-RFLAT-1 was injected into rabbits to generate polyclonal antibodies. Anti-RFLAT-1 antiserum was purified over a protein A column followed by an antigen column. For Western blots of transfected cells, cultures were harvested 36 hr after transfection and nuclear extracts were prepared according to Andrews and Faller (1991). For PBL and other cell lines, nuclear extracts were prepared as described above. Anti-RFLAT-1 Western blotting was performed as follows: membranes were blocked in 5% non-fat milk in TBST (0.02 M Tris-HCl [pH 7.4], 0.5 M NaCl, and 0.2% Tween-20), washed with TBST, and incubated in blocking buffer containing antigen purified anti-RFLAT-1 (1:1000 [v/v]). The membranes were then washed, incubated in blocking buffer with horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:10,000 [v/v]), washed, detected by ECL (Amersham, Arlington Heights, IL), and exposed to Amersham Hyperfilm. Anti-p50 and anti-p65 Western blotting was performed following the manufacturer's instructions. In vitro transcription/translation assays were performed using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI) following the instructions.

Immunostaining and EMSA

1×10^4 cells were adhered to 8-well slides (Electron Microscopy Sciences, Ft. Washington, PA) and fixed in methanol followed by acetone. The wells were incubated in blocking buffer (5% goat serum, 5% horse serum, and 0.1% Triton X-100 in PBS), washed in PBS supplemented with 0.1% Triton X-100, and incubated with antigen purified rabbit polyclonal anti-RFLAT-1 (1:500 [v/v]) or rabbit IgG (1:500 [v/v]) (Santa Cruz Biotechnology, Santa Cruz, CA). The wells were washed, incubated with goat anti-rabbit FITC (1:2000 [v/v]) (Caltag, Burlingame, CA) and anti-FITC Alexa 488 (1:2000 [v/v]) (Molecular Probes, Eugene, OR). Finally, the slide was washed with PBS, covered with mounting media (1:3 [v/v], Vectashield with propidium iodide) (Vector, Burlingame, CA), cover slipped, and sealed. EMSA and supershift assays were performed essentially as described by Ortiz et al. (1996). Oligonucleotides were synthesized by Life Technologies, Inc. (Gaithersburg, MD) and purified by a polyacrylamide-urea gel. Oligonucleotides were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The following oligonucleotides were used:

A/B, AATTCGCTATTTTGGAAACTCCCCTTAGGGGATGCCCCTC AACTGCG; A, AATTCGTTGCTATTTTGGAAACTCCCCTTG; B, AATT CGCTAGGGGATGCCCCTCAACTGCG; E, AATTCTTTGTGCAATTT CACTTATGATACCG; C, AATTCTCTAGATGAGAGAGCAGTGAGGG AGAGACG; and κB, AATTCGTCAGAGGGGACTTTCCGAGAG.

Anti-Sp1, anti-Sp3, anti-p65, and anti-c-rel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p50, anti-p65, and anti-α-actinin were from Upstate Biotechnology (Lake Placid, NY). Anti-NPCP was from BAbCO (Berkeley, CA).

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