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Kinetics of Transcription Factors Regulating the RANTES Chemokine Gene Reveal a Developmental Switch in Nuclear Events during T-Lymphocyte Maturation

BENJAMIN D. ORTIZ,[1] ALAN M. KRENSKY,[1,2]* AND PETER J. NELSON[2]†

Multidisciplinary Program in Immunology[1] and Division of Immunology and Transplantation Biology,[2] Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305-5119

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RANTES is a chemoattractant cytokine (chemokine) whose gene is expressed immediately after stimulation of several cell types but upregulated late (3 to 5 days) after activation in normal T lymphocytes. Here we describe two *cis*-acting elements in the human RANTES promoter that act in T lymphocytes. One site interacts with NFIL6, which is activated within the first 24 h after T-cell activation. The second site binds an apparently novel complex that is upregulated later, between days 3 and 5. These data provide an explanation for the immediate-early expression of RANTES in some cell types and identify apparently novel factors contributing to late RANTES transcription in T cells. The results reveal a developmental switch occurring during normal T-cell maturation coincident with the onset of terminal differentiation and the binding of late-acting factors to sequences of the RANTES promoter.

T-lymphocyte development is divided into discrete stages. The initial stages occur within the thymus and result in the release of selected T-cell populations into the peripheral blood. These T lymphocytes are fully competent to respond to specific peptide antigens bound to their self major histocompatibility complex molecules (37). Later stages of the developmental program begin in the periphery after a resting T cell is triggered through its antigen-specific receptor (47). This event irreversibly commits the activated T cell to a program of terminal differentiation into a specific effector cell, such as a cytolytic T lymphocyte (CTL) or a T helper cell (6). This maturation process takes 3 to 7 days and is characterized by the coordinated expression of new genes including interleukins (ILs), proto-oncogenes, and, days later, those genes involved in cytolysis and movement through tissues (46). Much has been learned about the genes expressed within hours of the initial activation of resting peripheral blood T cells. The study of one of these genes, the gene encoding IL-2, has provided important insights into the molecular basis of T-cell activation and identified the NFAT transcription factor family, thought to be pivotal mediators of T-cell commitment and the concomitant expression of many cytokine genes (22, 23, 30, 32, 35).

In contrast, little is known about the molecular mechanisms underlying the upregulation of genes expressed later after T-cell activation. Three to five days after initial stimulation of T lymphocytes with an antigen or mitogen, subsets develop cytolytic ability and express effector genes such as those encoding perforin and the granzymes (6, 34). Also upregulated in this 3- to 5-day window is the gene coding for the chemokine RANTES (41, 46). The RANTES gene encodes an 8-kDa cytokine that selectively chemoattracts T cells, monocytes, and eosinophils (17, 40). It is part of the large chemokine family whose members have chemoattractant and activating effects on different specific subsets of leukocytes (25, 39, 44).

Analogous to perforin and granzyme expression, RANTES mRNA expression is upregulated late and is maintained in terminally differentiated T-cell lines in culture, such as CTL (41). These kinetics are apparently unique to T cells, because many other cell types transiently upregulate RANTES mRNA rapidly, within hours after stimulation (7, 36, 42, 51). The expression patterns and functional properties of RANTES led us to hypothesize a fundamental role for RANTES in the inflammatory process (33, 48). After the early and transient activation of RANTES by cells in a stressed tissue, T lymphocytes are attracted to the site of inflammation where they can meet their cognate antigen, fully differentiate, and then produce large amounts of RANTES, thus amplifying and propagating the inflammatory response. Understanding the molecular basis of the regulation of RANTES mRNA in T cells may provide insight into the transcriptional regulatory machinery operating late in T-lymphocyte development and may yield new targets for manipulation of inflammatory responses.

In this report, we identify two *cis*-acting elements of the human RANTES promoter contributing to full activity in T lymphocytes and identify both an early-acting and a late-acting transcriptional regulatory pathway contributing to RANTES gene expression. These data, with other reports, explain how the gene is expressed early in some cell types and identifies a candidate transcription factor contributing to the late upregulation of RANTES expression in T lymphocytes.

MATERIALS AND METHODS

Cells and cell lines. HUT78 (ATCC TIB 161), Jurkat (ATCC TIB 152), Burkitt's B-lymphoma cell lines MS (50) and Daudi (ATCC CCL 213), PEER (a γδ T-cell line), and normal peripheral blood lymphocytes (PBL) were cultured in RPMI 1640 medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 2 mM L-glutamine, 100 U of penicillin G per ml, 100 U of streptomycin per ml, and 10% heat-inactivated fetal calf serum (HyClone Laboratories, Inc. Logan, Utah). YT2C2, a natural killer cell tumor (3), was cultured as described above, with sodium pyruvate added to a final concentration of 1 mM. Normal human CTL lines were generated and maintained as described previously (5). RD (ATCC

* Corresponding author. Mailing address: Division of Immunology and Transplantation Biology, Department of Pediatrics, Room 306, Stanford University School of Medicine, Stanford, CA 94305-5119. Phone: (415) 498-6073. Fax: (415) 498-6077.

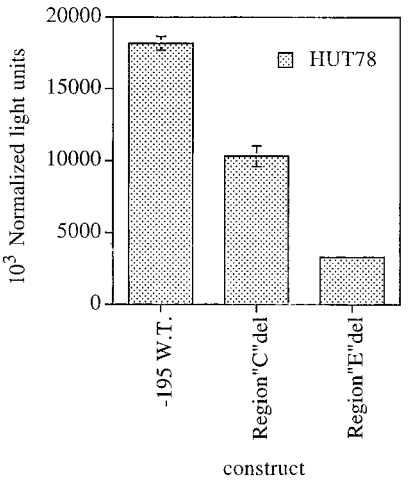
† Present Address: Arbeitsgruppe Klinische Biochemie, Medizinische Poliklinik, Ludwig-Maximilians-Universität, D-80336 Munich, Germany.

CCL 136), a rhabdomyosarcoma, was cultured in RPMI 1640–15% bovine calf serum supplemented with nonessential amino acids and vitamins. Normal dermal fibroblasts were cultured as described previously (43). SK-HEP-1 (ATCC HTB 52) cells were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin G per ml, and 100 U of streptomycin per ml. PBL were isolated by Ficoll density gradient centrifugation from buffy coats obtained from healthy blood donors at the Stanford Blood Bank. The viability of isolated PBL was greater than 99% as determined by trypan blue exclusion. PBL were activated with 5 μg of the T-cell mitogen phytohemagglutinin-P (PHA) (Difco, Detroit, Mich.) per ml. Only the nonadherent cells were harvested for experiments. This activated population was greater than 95% T cells as determined by flow cytometry by using a monoclonal antibody to CD3 (OKT3). All cells were cultured in horizontal flasks (LUX, Naperville, Ill.).

Plasmid constructs, transient transfection, and luciferase reporter gene assays. The construction of the various RANTES promoter luciferase reporter constructs has been previously described (28). The region C deletion was constructed by removing a *Sac*I-to-*Bsp*EI fragment from the −195 construct. The region E internal deletion was constructed by cloning a PCR (16)-generated fragment representing bases −115 to −195 upstream of a −91 RANTES promoter construct (created by deletion of a *Sac*I-to-*Msp*I fragment). To generate the −195 R(C)mutant, point mutations were introduced in region C of the −195 promoter by PCR with an oligonucleotide containing the specific G-to-T mutations indicated spanning the region to be altered and the GL2 oligonucleotide (Promega, Madison, Wis.). PCR products were cloned into pGL2 (Promega) by using *Sac*I and *Hin*dIII sites. The construct sequence was confirmed by dideoxy chain termination DNA sequencing (2). HUT78 cells were transfected by electroporation as described previously (28), with 5 μg of the indicated reporter constructs. Three micrograms of a reporter construct containing the cytomegalovirus immediate-early promoter driving the *lacZ* gene was cotransfected and used to normalize for transfection efficiency (gift of L. Naumovski, Stanford University). The C/EBPβ/NFIL6 expression construct (gift of S. Akira, Osaka University, through S. Kinoshita and G. Nolan, Stanford University) contained the cDNA in the pEFBOS expression vector (24, 26). Control pEFBOS was made by excising the cDNA by using *Xba*I and *Sal*I sites. Ten micrograms of the expression vector or control was used per replicate in the cotransfection assays. Luciferase assays were performed using the luciferase assay system kit (Promega) as described previously (28). β-Galactosidase assays were performed with an aliquot of transfected cell extracts according to the instructions accompanying the reporter lysis buffer reagent (catalog no. E397A; Promega) with *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The results were recorded on a Beckman DU62 spectrophotometer set at a wavelength of 420 nm. All constructs were tested at least in triplicate. The data presented are representative of more than three separate experiments. Only plasmids prepared at the same time were directly compared in reporter gene assays, and all results reported were confirmed with at least two separate plasmid preparations.

Preparation of nuclear extracts, EMSA, and methylation interference assays. Nuclear proteins were prepared essentially according to the protocol of Durand et al. (8), except 0.2% Nonidet P-40 was used in buffer A to lyse the cells instead of the homogenizer. Extracts were desalted of ammonium sulfate with a P6DG resin (Bio-Rad, Hercules, Calif.) and quantitated by the Bradford assay using the Bio-Rad protein assay reagent. The electrophoretic mobility shift assay (EMSA) was performed essentially as described previously (8). Briefly, binding reaction mixtures (15 μl, final volume) contained 10 mM Tris-Cl (pH 7.5), 80 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 1.5 μg of poly(dI · dC), 5 to 10 μg of nuclear extract, and 20 kcpm (0.1 to 0.5 ng) of the ^{32}P-end-labeled double-stranded oligonucleotide probe. After incubation for 45 min on ice, the protein-DNA complexes were resolved on nondenaturing 5% polyacrylamide gels run in 1× Tris-borate-EDTA (TBE) buffer (2). Oligonucleotides were synthesized by Genset (San Diego, Calif.), with 5′ overhangs that could be end labeled by the Klenow fragment as described previously (2, 8). For cold oligonucleotide competition assays, a 1,000-fold molar excess of unlabeled DNA was added to the binding reaction mixture as indicated prior to the addition of the labeled probe. For antibody supershift-blocking assays, the antibody reagents were added to the gel shift reaction mixture 15 min into the incubation, and the mixture was further incubated for 30 min at 4°C prior to gel loading. C/EBP family antisera (α, β, and γ) were purchased (Santa Cruz Biotechnology, Santa Cruz, Calif.) and used according to the manufacturer's instructions. The ets family antisera was a gift (R. Fisher, National Cancer Institute Frederick, Md.) (38). Methylation interference was assayed as described elsewhere (2). Preparative EMSA (10-fold scale up of reaction described above) was performed using the C region *Sac*I-to-*Bsp*EI single-end-^{32}P-labeled restriction fragment. The fragment was labeled with the Klenow fragment. DNA was eluted from the excised bands representing EMSA complexes by electroelution in a Bio-Rad apparatus. Following piperidine cleavage, the DNA ladders were analyzed on standard 10% polyacrylamide–urea sequencing gels (2).

UV cross-linking analysis. Preparative EMSA was performed exactly as described for methylation interference. Before autoradiography, the gel was exposed to UV light (2,500 mJ) in a Stratalinker (Stratagene, La Jolla, Calif.) as described previously (18). Bands were excised and heated to 70°C in Laemmli sample buffer. Gel slices were then loaded into the wells of a sodium dodecyl sulfate–10% polyacrylamide electrophoresis (SDS–10% PAGE) gel run in Tris-glycine-SDS buffer (2). Molecular weight standards were ^{14}C-labeled Rainbow markers (Amersham).



Region "C" sequence:-195 to -144
GAGCTCACTCTAGATGAGAGAGCAGTGAGGGAGAGACAGAGAC
TCGAATTT

Region "E" sequence: -115 to -91
T**TTTGTGCAAT**TTCACTTATGATACC

FIG. 1. Luciferase reporter gene assays of HUT78 T cells using −195 wild-type (W.T.) RANTES promoter and indicated sequences deleted (del) internally. NFIL6 binding site homology is underlined. The results are presented as normalized (to cotransfected cytomegalovirus promoter β-galactosidase reporter construct) light units. The absence of an error bar indicates an error too low to be recognized by the graphing program.

RESULTS

Identification of two *cis*-acting elements that contribute to RANTES promoter activity in HUT78 T-cells. HUT78 T-cell leukemia cells constitutively express the RANTES gene (28) in addition to the genes encoding IL-2, IL-2Rα (12), perforin, other cytolytic granule proteins characteristic of activated, mature T lymphocytes (13). Deletion analysis of the RANTES promoter identified two regions upstream of the CCAAT box which are important for optimal RANTES promoter-driven reporter gene expression in T lymphocytes (Fig. 1). The deletion of sequences from −195 to −144, denoted region C, reduced RANTES promoter activity by 40 to 60%. Similarly, an internal deletion of sequences from −115 to −91, designated region E, resulted in a loss of as much as 80% of reporter gene activity in HUT78 cells. Region D (−144 to −115), homologous to a pyrimidine-rich sequence containing lipopolysaccharide-responsive elements functionally identified in murine macrophage cell lines (42), did not appear to contribute to promoter activity in HUT78 cells (data not shown). While region E contains a consensus binding site for NFIL6/C/EBPβ (Fig. 1) (10), region C did not contain sequences with striking homology to any known transcription factor binding site consensus sequence, as determined by visual comparison by using the information in reference 10.

Identification of nuclear factors interacting with the C region and definition of their recognition sequences. HUT78-derived nuclear factors form a single complex on DNA from region C when tested in EMSA (Fig. 2A). A similar complex was found in other cell lines including MS, a B-cell (Burkitt's lymphoma) tumor line. Jurkat, a T-cell leukemia line with a resting phenotype, expressed it at a much lower level. Levels of

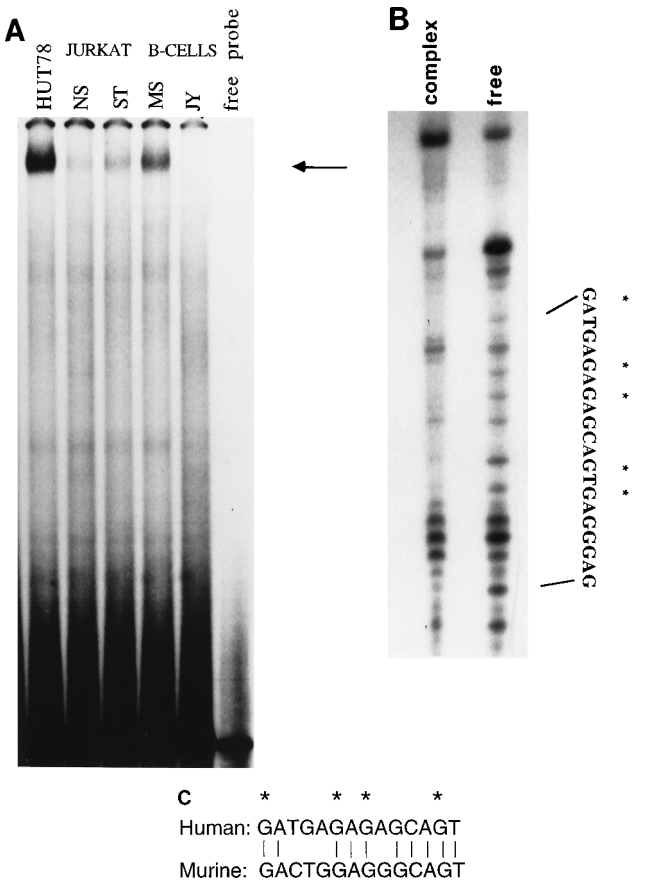


FIG. 2. Definition of the binding site for nuclear factors recognizing region C. (A) EMSA using ^{32}P-labeled restriction fragment containing region C. The arrow indicates the major complex. Jurkat T cells were stimulated with 20 ng of PMA per ml and 2 μM ionomycin for 2 h. (B) Methylation interference assay using the same restriction fragment. Undermethylated G residues (contact residues) are indicated by asterisks. Complex, bound probe; free, unbound probe. (C) Comparison of human and murine RANTES promoters at the binding site. Asterisks indicate contact residues. The 5′ border nucleotide positions are −187 (human) and −169 (murine) relative to the transcription start site (+1).

this complex were not altered by stimulation of Jurkat for 2 h with the phorbol ester phorbol 12-myristate 13-acetate (PMA) and ionomycin under conditions known to upregulate transcription factors such as NFAT, NFκB, and AP-1 (9).

A methylation interference assay was employed to identify the binding site recognized by this complex (Fig. 2B). Dimethyl sulfate partially methylated probes recruited into the EMSA complex by region C binding nuclear factors were purified alongside the unbound probe. Comparison of piperidine cleavage ladders generated from both pools of probe revealed a series of undermethylated G residues spanning 15 nucleotides on probes involved in EMSA complex formation. The position and sequence of this purine rich region, designated the R(C) site, are highly conserved between the murine and human RANTES promoter (Fig. 2C) (7, 28).

Mutation of contact G residues of the R(C) site abolishes nuclear factor binding and impairs RANTES promoter activity. To confirm the results of the methylation interference assay, an oligonucleotide which spanned the 15-nucleotide sequence identified by methylation interference plus the immediate four bases 5′ and 3′ to the site was synthesized. A mutant R(C) site oligonucleotide, designated R(C)-M, was also made by altering the G nucleotides identified as contact residues to T bases. When these oligonucleotides were tested in EMSA (Fig. 3A), the R(C) site oligonucleotide produced a single band complex with HUT78 nuclear protein similar to that formed on the entire region C fragment. The mutated oligonucleotide failed to support any complex formation in EMSA. Moreover, identical G-to-T point mutations introduced into the RANTES promoter impaired its activity to the same extent as the complete C region deletion, indicating that nuclear factors interacting with the R(C) site are responsible for the transcriptional activity observed in the reporter gene assays (Fig. 3B).

To confirm that the R(C) site binding complex was capable of binding the entire region C DNA, cross-competition EMSA experiments were performed. Unlabeled region C DNA efficiently competed for the R(C) binding complex (data not shown). Although the data indicate that the R(C) site binding complex is responsible for most, if not all, of the transcriptional activity associated with region C, there is additional evidence for interactions between DNA-binding proteins and other sequences in this region. However, at this point, the functionality and sequence specificity of these interactions are unclear.

R(C) site binding protein activity is widespread but highly expressed in lymphoid cell lines. Nuclear extracts prepared from cell lines of various lineages were tested for R(C) site binding protein activity (Fig. 4A). High levels of R(C) complex activity were found in normal PBL activated with PHA for 5 days, the lymphoid tumor cell lines HUT78, PEER (a γδ leukemia T-cell line), and two Burkitt's lymphoma (B-cell) lines, MS and Daudi. Intermediate levels of expression were found in

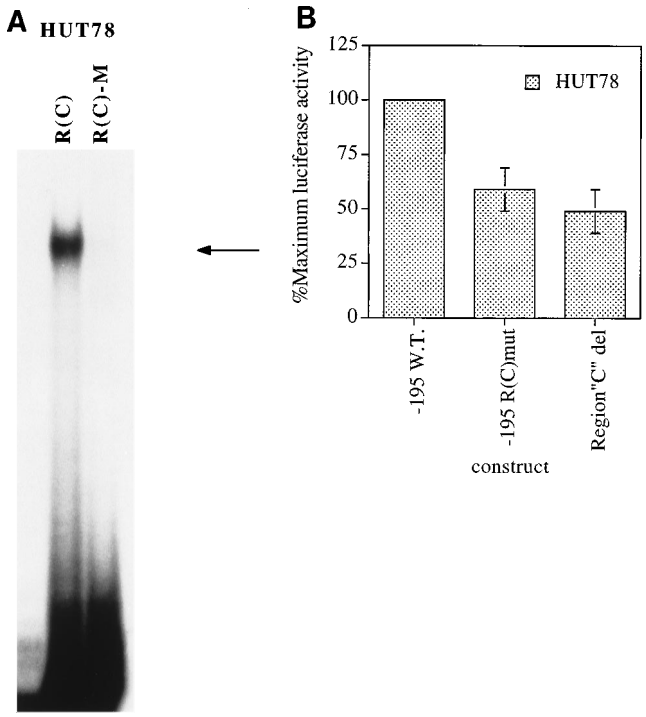


FIG. 3. R(C) site point mutations eliminate nuclear factor binding and impair RANTES promoter activity. (A) EMSA with ^{32}P-labeled R(C) oligonucleotide (sequences −187 to −164) and R(C)-M [same as R(C) except G residues indicated by asterisks in Fig. 2B are altered to T). (B) Luciferase reporter gene assays of HUT78 T-cells with the −195 wild-type (W.T.) promoter. The −195 R(C)mutant (mut) is identical to −195 except that the same G-to-T mutations as described for the R(C)-M oligonucleotide in panel A were introduced. The region C deletion is described in the legend to Fig. 1. The results are the averages for two experiments using two different sets of plasmid preparations.

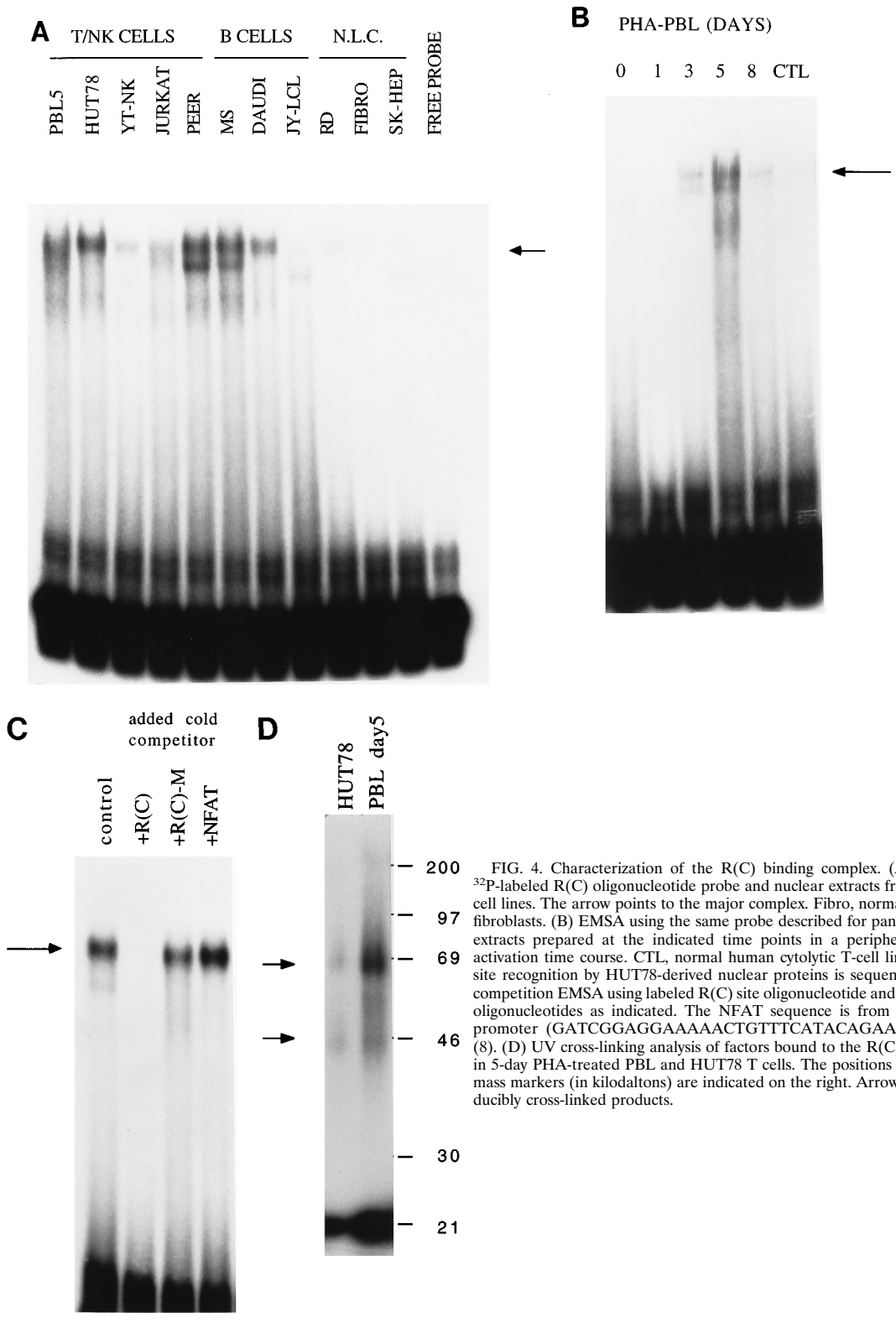


FIG. 4. Characterization of the R(C) binding complex. (A) EMSA using ^{32}P-labeled R(C) oligonucleotide probe and nuclear extracts from the indicated cell lines. The arrow points to the major complex. Fibro, normal human dermal fibroblasts. (B) EMSA using the same probe described for panel A and nuclear extracts prepared at the indicated time points in a peripheral blood T-cell activation time course. CTL, normal human cytolytic T-cell line (5). (C) R(C) site recognition by HUT78-derived nuclear proteins is sequence specific. Cold competition EMSA using labeled R(C) site oligonucleotide and unlabeled excess oligonucleotides as indicated. The NFAT sequence is from the human IL-2 promoter (GATCGGAGGAAAAACTGTTTCATACAGAAGGCGTGATC) (8). (D) UV cross-linking analysis of factors bound to the R(C) oligonucleotide in 5-day PHA-treated PBL and HUT78 T cells. The positions of the molecular mass markers (in kilodaltons) are indicated on the right. Arrows point to reproducibly cross-linked products.

Jurkat and YT2C2 (natural killer tumor cell line). Expression was low to undetectable in a third B-cell line, JY, and the nonlymphoid cell lines, RD, a rhabdomyosarcoma (muscle cell) line, normal human dermal fibroblasts, and the endothelial cell tumor line SK-HEP. Binding activity was also detected in U937, a monocytic cell line, and in HepG2, a hepatoma cell line (data not shown). An additional band, which migrated slightly faster in EMSA, was found in the MS and PEER lanes.

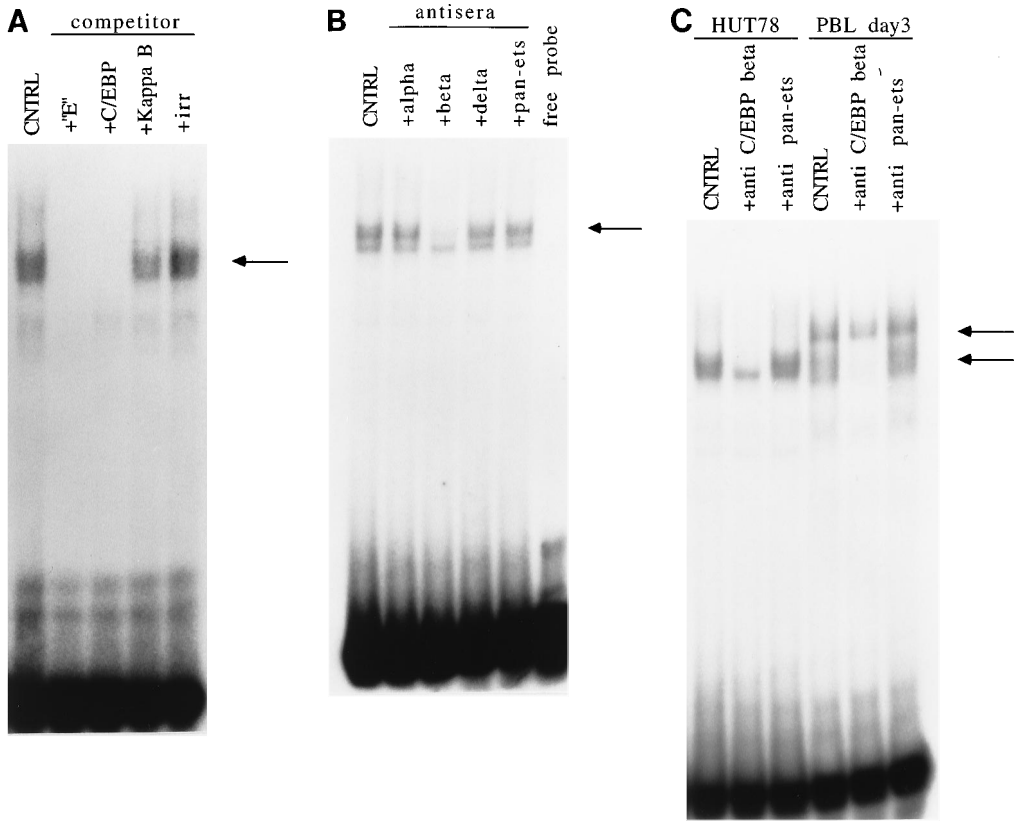


FIG. 5. The region E binding complex contains NFIL6/C/EBPβ. (A) EMSA using ^{32}P-labeled region E oligonucleotide and HUT78 nuclear extract. Cold oligonucleotide competitors were used in 1,000-fold molar excess. "E," homologous oligonucleotide; C/EBP, C/EBP consensus binding site (Santa Cruz Biotech catalog no. sc-2525); Kappa B, NF-κB binding sequence from immunoglobulin kappa light-chain enhancer (TCGAGTCAGAGGGGACTTTCCGAGTCGA) (49); irr, irrelevant sequence oligonucleotide (GATCCTGGAAGGGAGAGTGGAGATC). (B) EMSA-antibody supershift/blocking assay using the probe and extracts described for panel A. Rabbit polyclonal immunoglobulin G (1 μg) was added as described in Materials and Methods. Alpha, beta, and delta refer to the specific C/EBP family members against which the antisera are directed (Santa Cruz Biotech). The arrow points to the blocked EMSA complex. (C) Similar to the assay described for panel B but with nuclear extracts from both HUT78 T cells and PBL 3 days after PHA treatment. Antisera were added as indicated. The lower arrow points to the C/EBPβ/NFIL6 complex. The upper arrow indicates the additional late PBL-derived EMSA complex. CNTRL, control.

This binding activity varied among experiments and may be that of an R(C) binding complex degradation product.

C site binding protein is upregulated late after T-cell activation. PBL from healthy human donors were activated with the T-cell mitogen PHA to mimic antigen stimulation. Nuclear extracts were isolated at time zero and 1, 3, 5, and 8 days following activation. The R(C) site binding complex was strongly, but transiently, upregulated between days 3 and 5 (Fig. 4B). Additional time course experiments have shown that levels of this complex remain high at least through days 4 to 6 (data not shown). These kinetics are unusual since induction of most known transcription factors occurs within the first 24 h after T-cell activation (46). The timing of R(C) complex upregulation is coincident with the late upregulation of RANTES mRNA in normal T cells (41). We will hereafter refer to this complex as R(C)FLAT for RANTES C site binding factor of late-activated T cells.

R(C)FLAT is an apparently novel complex with at least two DNA binding subunits. In order to characterize the components of the R(C)FLAT complex, competition assays were performed using excess cold oligonucleotides representing known purine-rich transcription factor binding sites. R(C) binding activity was specifically inhibited by the homologous oligonucleotide but not the mutant R(C)-M oligonucleotide nor the purine-rich NFAT site of the human IL-2 promoter (8) (Fig. 4C). Further, the binding detected in the other R(C) complex-positive cell types was similarly found to be sequence specific by EMSA competition assays (data not shown). R(C) binding was not inhibited by using consensus binding sites for AP-1, NFκB, C/EBP, NFIL6, Oct-1, or ets family transcription factors (data not shown) (10).

To further characterize the DNA binding subunits of R(C) FLAT, the complex was resolved on EMSA and subjected to UV cross-linking. After exposure to X-ray film, the EMSA complex band was excised, treated as outlined in Materials and Methods, and run on an SDS-PAGE gel (Fig. 4D). Two proteins, which migrated at approximately 65 and 45 kDa, were reproducibly cross-linked to the R(C) site. Subtracting the mass of the cross-linked DNA (17 kDa), these proteins have apparent molecular masses of approximately 48 and 28 kDa, respectively. These cross-linked products were found in both HUT78 T-cells and in 5-day PHA-activated PBL.

Identification of nuclear factors binding to region E by cold oligonucleotide competition and antibody supershift/blocking assays. HUT78-derived nuclear factors interacting with a region E oligonucleotide, which includes the region of NFIL6 binding site homology, formed a doublet of complex bands on EMSA (Fig. 5A). This doublet was specifically inhibited by an excess of cold homologous competitor oligonucleotide or consensus C/EBP binding site oligonucleotide. A kappa B site and another irrelevant oligonucleotide were not able to inhibit this complex.

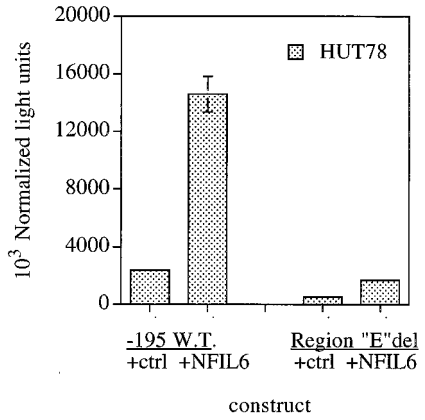


FIG. 6. Luciferase reporter gene assay using HUT78 T cells. Reporter constructs −195 W.T. and region E deletion (del) are described in the legend to Fig. 1. Cotransfected expression constructs were added as described in Materials and Methods. Ctrl designates the pEFBOS expression vector (without the cDNA). NFIL6 indicates the pEFBOS expression vector containing NFIL6 cDNA. The absence of error bars indicate errors too low to be recognized by the graphing program.

To determine if these complexes were formed by C/EBP family proteins, supershift/blocking antisera against C/EBPα, C/EBPβ/NFIL6, and C/EBPδ/NFIL6β were tested to identify the complex (Fig. 5B). Only the C/EBPβ/NFIL6 antiserum affected the binding pattern by preventing the formation of the upper complex of the doublet. A control antiserum to ets family proteins had no effect on the EMSA pattern. To confirm the presence of NFIL6 in normal T cells, extracts from day-3 PHA-activated PBL were tested in the supershift assay alongside the HUT78 nuclear extracts (Fig. 5C). The activated PBL proteins formed a doublet similar to, but less distinct than, that found in HUT78. Furthermore, this complex was also blocked by the C/EBPβ/NFIL6 antiserum. An additional upper band was found in the activated PBL EMSA pattern but not in the HUT78 lanes. This complex was not affected by the C/EBPβ/NFIL6 antiserum. Antibodies to C/EBPα and C/EBPδ did not affect the activated PBL-derived EMSA pattern (data not shown).

Cotransfection/reporter gene assays with C/EBPβ/NFIL6 expression vector. To confirm that NFIL6 could activate transcription through region E, an expression vector containing the NFIL6 cDNA under control of the elongation factor promoter was cotransfected with the RANTES −195 promoter-luciferase construct. The parent expression vector without the cDNA was used as a control. The NFIL6 cDNA stimulated RANTES promoter driven activity over sixfold in HUT78 T-cells. Activity from a reporter construct with region E internally deleted was only minimally enhanced by the NFIL6 expression vector (Fig. 6).

Kinetics of NFIL6 upregulation after normal T-cell activation. By using the same nuclear extracts as those used for the experiment shown in Fig. 4B, the kinetics of NFIL6 induction in activated T cells were examined. Unlike the R(C)FLAT complex, the NFIL6 complex is upregulated by day 1 of the time course and is nearly absent by day 5. The upper band formed by PBL nuclear extracts was upregulated later, by day 3, and was maintained throughout the remainder of the time course. It was also present in terminally differentiated normal cytotoxic T cells (Fig. 7). We will refer to this as the E region binding FLAT.

DISCUSSION

The RANTES gene is upregulated late (3 to 5 days) after normal peripheral blood T-cell activation and is constitutively expressed in a T-cell tumor with an activated phenotype, HUT78. In contrast, it is also expressed as an immediate-early gene in many other cell types, including macrophages, fibroblasts, and renal mesangial and tubular epithelial cells in response to specific stimuli (7, 36, 42, 51). This study of RANTES promoter activity in HUT78 and the nuclear factors regulating the RANTES promoter in HUT78 and normal T cells provides an explanation for immediate-early expression in some cells. In addition, candidate factors contributing to the late upregulation of RANTES transcription in normal T cells have been identified.

The NFIL6/C/EBPβ transcription factor positively regulates the RANTES promoter in HUT78 T cells by interacting with region E sequences. Since it is upregulated early after PBL activation, the physiologic relevance of NFIL6 in RANTES gene expression in normal T cells is still unclear. This binding may help to explain why there is sometimes a low, early, and transient upregulation of RANTES mRNA in response to PHA (41). NFIL6 is known to respond within hours to cytokine-mediated signal transduction (1). Therefore, it, in combination with factors binding the lipopolysaccharide-responsive element sequences contained in region D (by others studying murine macrophages [42]), provides a molecular basis for immediate-early expression in a wide variety of cell types. Our previous finding of functional Rel/NF-κB family protein binding sites A and B (discussed below) in the promoter indicate that these proteins might also contribute to RANTES upregulation in some of these cell types (4, 31).

However, additional factors present in late-activated normal

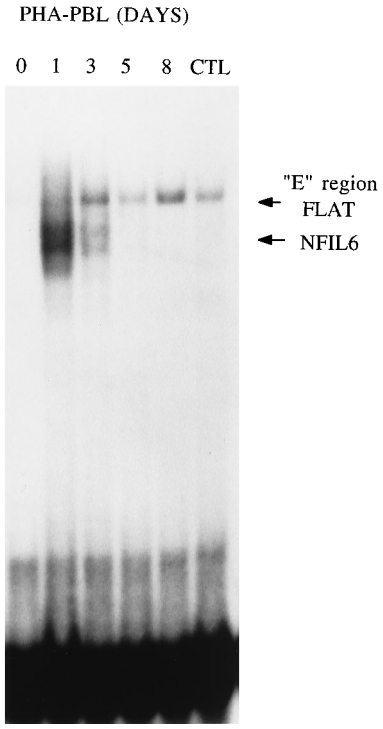


FIG. 7. EMSA using ^{32}P-labeled region E oligonucleotide and the same extracts described in the legend to Fig. 4B. "E" region FLAT denotes the late PBL-derived EMSA complex. The NFIL6 arrow indicates the complex C/EBPβ/NFIL6 as determined by the antibody blocking assay.

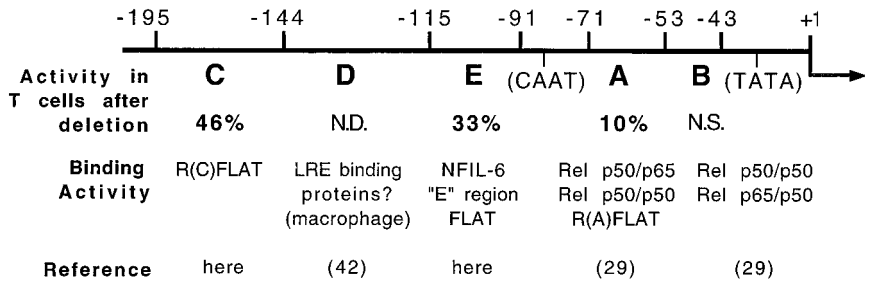


FIG. 8. Diagram of regulatory regions identified in the −195 RANTES promoter and their positions relative to transcription start site +1. The percentages of activity in T cells after the deletions are relative to the −195 wild-type activity and are averages derived from the number of experiments per region: for C, $n = 14$; for E, $n = 7$, and for A, $n = 4$. The binding activities identified within various regions are also listed. N.D., not determined; N.S., not significantly altered.

T cells cause the upregulation of RANTES mRNA after the immediate-early factors have been downregulated. This report identifies a transcription factor complex, R(C)FLAT, that activates the RANTES promoter through a purine-rich R(C) site. This factor is induced between days 3 and 5 after initial T-cell activation, coincident with the late upregulation of RANTES mRNA. This complex contains at least two DNA binding subunits and does not appear to be related to several transcription factor families known to bind purine-rich sequences. Although R(C)FLAT is highly expressed in many lymphoid cell lines, R(C)FLAT expression does not perfectly correlate with RANTES mRNA expression. The R(C)FLAT-positive cell lines Jurkat, PEER, MS, and Daudi do not express RANTES. Therefore, this transcription factor is not an absolute determinant of RANTES gene expression. Preliminary data indicate that transcriptional regulation through the R(C) site may be context dependent. Neither the R(C) site nor region C were capable of *trans*-activating a heterologous simian virus 40 basal promoter (data not shown). This is also a property of the T-cell receptor beta enhancer which is much more efficient at transactivating its own promoter than a heterologous one (19). In addition, it is reminiscent of the context-dependent transcriptional regulatory protein lymphoid enhancer factor 1 whose binding site is found in the T-cell receptor alpha enhancer (11). Since lymphoid enhancer factor 1 is expressed early in T-lymphocyte ontogeny (45), it is unlikely to be R(C)FLAT.

We have identified another late-activated transcription factor complex, R(A)FLAT which acts through a downstream kappa-B-like site (29). R(C)FLAT and R(A)FLAT mediate transcriptional control mechanisms likely to contribute to late upregulation of RANTES mRNA in normal T cells. In addition, the E region binding FLAT complex may also play a role in this process. It is also upregulated on day 3 of T-cell activation and is maintained by fully differentiated cytotoxic T cells, which express RANTES constitutively (41). Formal demonstration of its role in RANTES gene expression, however, awaits further characterization of the proteins forming this EMSA complex.

In identifying two additional functional regions of the RANTES promoter, there are now four identified *cis*-acting elements contributing to RANTES transcription in various systems. Regions A through E (Fig. 8) comprise a promoter with the capacity to respond to different microenvironmental and developmental stimuli. Region A binds proteins of the Rel family (31) and the late-activated R(A)FLAT complex (29). Region B was not found to contribute significantly to activity in T cells but can also bind members of the Rel family (29). Region C contains a purine-rich sequence that binds the R(C) FLAT complex. Region D, highly conserved between murine and human promoters (27), contains sequences described as a lipopolysaccharide-responsive element in murine macrophages (42). Finally, region E, also described here, binds the well-characterized NFIL6 transcription factor and another, as yet uncharacterized, late-activated factor in normal T cells. As opposed to the data for the single experiment presented in Fig. 1, the percentages of activity remaining after deletion or mutation of regions C and E represent averages of multiple experiments. Additional functional regions may be found in the RANTES promoter, underlying the regulation of its transcription in other cell types (28).

Identification of late-activated transcriptional control pathways that regulate RANTES may lead to an understanding of the mechanisms that propagate the inflammatory responses at sites of injury. It also illuminates a poorly understood stage of peripheral T-cell development. By using the RANTES promoter as a functional probe of transcriptional regulatory pathways in HUT78 T-cells, we have identified transcription factors that are uniquely regulated during normal peripheral blood T-cell activation. The data presented here suggest a switch in the transcription factor sets utilized in T lymphocytes during the transition from early-to-late activation processes. Figure 9

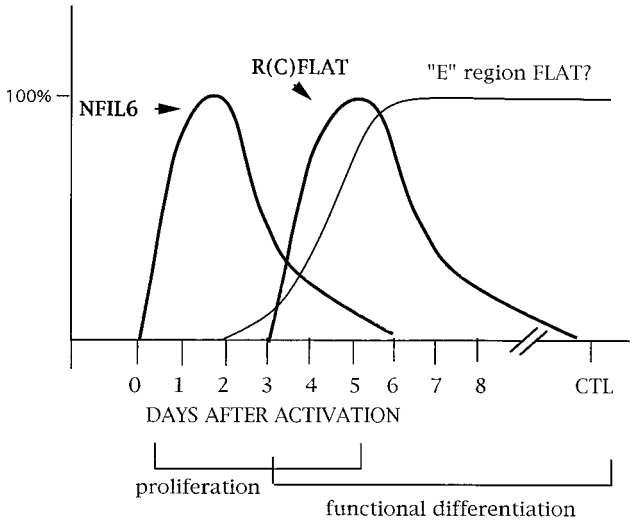


FIG. 9. Temporal expression of transcription factors regulating the RANTES promoter demonstrates a switch from transcription factors that are activated early (days 1 to 3) to those which are activated later (days 3 to 8) during peripheral blood T-cell development. The proliferation and functional differentiation stages are defined in reference 46. "E" region FLAT is represented as a thinner line pending further characterization of its contribution to RANTES gene expression.

depicts the general trend of RANTES promoter binding transcription factor kinetics during T-lymphocyte development. Early factors, such as NFIL6, with AP-1 (*fos*/*jun*) and NFAT, are upregulated within 24 h after T-cell activation (6, 46). In contrast, at days 3 to 5 postactivation, R(C)FLAT and R(A)FLAT (29) appear important for mediating late expression of RANTES. The timing of this switch is coincident with that period of T-cell development in which T-cell subsets acquire effector functions such as cytotoxicity. Newly expressed genes in this 3-to-5-day window include perforin and granzymes A and B (46). The protein products of these genes are thought to carry out aspects of cytolytic function in T cells and natural killer cells (34). Despite the functional importance of these late expressed proteins, very little is understood about the specific mechanisms controlling the expression of their genes (14, 15, 18, 21). The human perforin promoter region has an R(C)-related site at −121 (**G**ATGA**GG**CTGA**G**G, with conserved contact residues indicated in bold), but its function has not been determined (20). To date, no transcriptional regulatory pathways have been defined in normal T cells whose kinetics coincide with the onset of mRNA production from these loci. This work provides a first look at the transcriptional regulatory machinery operating at this important stage of T-cell development.

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