Identification of a Novel Regulatory Region Critical for Expression of the RANTES Chemokine in Activated T Lymphocytes[1]

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The RANTES chemokine is a T cell-expressed, proinflammatory cytokine recently implicated as a suppressive agent of HIV replication. We have identified tandem κB-like sequences within the promoter for RANTES that are critical for RANTES promoter-reporter gene activity in both the T cell tumor line Hut78 and in PHA-activated PBL. This region binds not only Rel family members (including p50-p65 heterodimers and p50-p50 homodimers) but also non-Rel factors up-regulated in PBL 3 to 5 days following activation. The expression of these "late" expressed nuclear factors correlates with an up-regulation of RANTES message found at this point in T cell activation. These factors are also constitutively expressed in functionally mature CD8$^+$ T cells. We hypothesize that these apparently novel proteins are responsible in part for the temporal regulation of RANTES seen in peripheral blood T cells and represent a component of transcriptional regulatory machinery newly expressed at this "late" stage of peripheral T cell development. *The Journal of Immunology,* **1996, 157: 1139–1148.**

RANTES[3] is a member of a growing family of immunoregulatory cytokines called chemokines, which function as chemotactic agents, induce changes in integrins, and activate specific effector cell populations (1, 2). RANTES is part of the C-C chemokine subfamily (1, 2) and was originally identified as a cDNA during a general screen for genes expressed by CD8$^+$ T lymphocytes (3). The RANTES chemokine has been implicated as a pivotal mediator of the inflammatory response and has recently been demonstrated to suppress replication of HIV (4). RANTES is a potent chemotactic agent for eosinophils, basophils, monocytes, NK cells, and CD45RO$^+$ "memory" CD4$^+$ T lymphocytes (1, 2, 5–10). It promotes the adhesion of CD4$^+$ T cells to activated endothelium, is released by thrombin-activated platelets, and causes activation of basophils, eosinophils, and T cells (2, 5, 6, 8, 11). These observations implicate RANTES in both acute and chronic phases of inflammation.

CD8$^+$ T cell suppression of HIV replication is well documented (12, 13). Soluble factors derived from CD8$^+$ T cells limit the ability of HIV to copy itself. This HIV-suppressing activity of CD8$^+$ T cells may be one part of the phenomenon of the "slow progressor" phenotype in some HIV-infected individuals (12, 13). Recently, the chemokines RANTES, MIP-1α, and MIP-1β produced by CD8$^+$ T cell were found to inhibit HIV replication in vitro (4). Understanding the control of expression of these chemokines in T cells will open new avenues of research into the pathogenesis of AIDS and the treatment of HIV-infected individuals.

The expression of RANTES can be induced in a variety of cell types (1, 2). In T cells, the expression of RANTES appears to be, in part, a developmentally controlled event in which strong up-regulation occurs 3 to 5 days after the activation of resting peripheral blood T cells with either mitogen or Ag (3, 14). This RANTES up-regulation occurs at a point in T cell maturation characterized by the expression of other "effector function" genes (15). In CD8$^+$ T cells, these include genes that encode the granzyme proteins and perforin (15).

We previously described the cloning and partial characterization of the human RANTES promoter in various cell types including the Hut78 T cell line (14, 16). In this report, we describe a complex control region within the RANTES promoter that is central to the transcriptional regulation of RANTES in T lymphocytes. This region, site R(A/B), binds Rel proteins and potentially novel transcription factors newly expressed by T lymphocytes 3 to 5 days following the activation of resting PBL. These novel factors are also expressed in chronically activated CD8$^+$ T cells.

Materials and Methods

Cells

PBL were isolated by Ficoll-Hypaque density gradient centrifugation from buffy coat material obtained from the Stanford Blood Bank, Stanford, CA or from the Amtlicher Blutspendedienst, Munich, Germany. PBLs were suspended at 2 to 4 $\times$ 10^6 cells/ml in tissue culture medium (RPMI 1640 supplemented with L-glutamine, penicillin, streptomycin, and 20% heat-inactivated FCS). Adherent cells (monocytes) in each preparation were reduced by incubation for 2 h at 37°C in 175-ml horizontal flasks (Lux, Naperville, IL). Nonadherent cells were then transferred to a new 175-ml horizontal flask for subsequent experiments. Viability of isolated PBL was greater than 99% as determined by trypan blue exclusion and was monitored through the course of the experiments. The PBL were activated with 5 μg/ml PHA-P (DIFCO, Detroit, MI) and incubated at 37°C for varying lengths of time. Cytotoxic CD8$^+$ T cell lines (CTL) were generated and

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Received for publication February 8, 1995. Accepted for publication May 13, 1996.

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[1] P.J.N. was a recipient of a National Institutes of Health Individual Research Service Award and is funded by the Sonderforschungsberich 1586. B.D.O. is a Howard Hughes Medical Institute Predoctoral Fellow. J.M.P. is a fellow of the National Kidney Foundation. A.M.K. is a Burroughs Wellcome Scholar in Experimental Therapeutics.

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[3] Abbreviations used in this paper: RANTES, regulated upon activation, normal T cell expressed, presumed secreted; R(A)FLAT, RANTES site R(A)-derived factors of late-activated T cells; NF-κB, nuclear factor-κB; EMSA, electrophoretic mobility shift assay; MIP-1α, β, macrophage inflammatory protein-1α, β.

0022-1767/96/$02.00

carried using established procedures (17). The T cell lines Hut78 (American Type Culture Collection (ATCC) TIB 161) and Jurkat (ATCC clone E61 TIB 152); RD (ATCC CCL 136), a rhabdomyosarcoma cell line; and MS, a Burkitt's (B cell) lymphoma cell line (18), were maintained in tissue culture medium as described above but with 10% heat-inactivated FCS. Normal dermal fibroblasts were obtained from Dr. E. Mocarski (Stanford University, Stanford, CA) and carried as described (19) for up to 10 passages, then were activated for 4 h with 10 ng/ml of TNF-α (R&D Systems, Minneapolis, MN) to induce nuclear factor-κB (NF-κB).

Transient transfection and luciferase reporter gene assays

A series of constructs derived from a 1024 nucleotide *Xho*I, *Kpn*I DNA fragment containing the immediate upstream region of the RANTES gene were subcloned into the pGL-3 vector (Promega, Madison, WI) as described (14, 16). Site-specific mutants were made using PCR and specific oligomers (20). The resultant constructs were DNA sequenced. The pGL-2 Pro vector (Promega) was utilized for enhancer activity assays.

Hut78 cells and activated PBL were transfected as described (14, 16) using a Bio-Rad Electroporator electroporation apparatus (Bio-Rad, Hercules, CA) according to the manufacturer's specifications. The electroporation voltage for the Hut78 cells was 240 V, and 270 V for the PHA-activated PBL, at 960 μF. In each experiment, 5 μg (for Hut78) to 20 μg (for PBL) of the individual reporter gene construct were co-transfected per replicate with 3 μg (for Hut78) to 10 μg (for PBL) of a control reporter construct containing the Rouse sarcoma virus promoter driving the β-galactosidase gene (Waltraud Ankenbauer, Heidelberg, Germany) (21). Luciferase assays were performed using the luciferase assay system kit (Promega) and a Lumat LB9501 Luminometer (Berthold, Wildbad, Germany). β-Galactosidase assays were performed on an aliquot of transfected cell extracts according to the instructions accompanying the Reporter Lysis Buffer Reagent (Promega item No. E397A) using *o*-nitrophenyl galactoside or using the Galacto-Light assay kit (Tropix, Bedford, MA). Results were read on a Beckman DU62 spectrophotometer set (Beckman Instruments, Fullerton, CA) at a wavelength of 420 nM or, in the case of the Galacto-Light system, a Lumat LB9501 Luminometer (Berthold). Each experiment was conducted at least in triplicate and the values were averaged after normalization to β-galactosidase expression and protein extract levels. Protein extracts were quantitated using Bio-Rad protein assay reagent (catalog No. 500-0006, Bio-Rad Laboratories, Munich, Germany). Correctness of all constructs was confirmed by DNA sequencing. Cell viability was followed through the course of the experiment using trypan blue exclusion and absolute cell number. Results routinely demonstrated 50 to 60% viability after initial electroporation and the cells showed approximately one doubling before assay for each of the constructs tested. Data presented are representative of at least three separate experiments. Only plasmids prepared at the same time were directly compared in reporter gene assays, and all results reported were confirmed across at least two separate plasmid preparations.

Isolation of nuclear extracts, electrophoretic mobility shift assays (EMSA), and supershift assays

Nuclear extracts for DNAse I footprinting, EMSA, and methylation interference assays were prepared according to the protocol of Durand et al. (22), with minor modifications as described by Ortiz et al. (14). EMSA were performed as described (14, 22, 23). Oligonucleotides used in EMSAs were synthesized by Genset (San Diego, CA) with *Xho*I and *Sal*I overhangs to allow end-labeling as described (24).

In EMSA competition assays, cold competitor oligomers at the specified concentration were added to the gel shift mixtures prior to addition of the ^{32}P-labeled oligonucleotide probe. For supershift EMSA, the antisera/mAb reagents were added to the gel shift mixture 30 min after initiating the incubation of the assay and the mix was further incubated for 30 min at 4°C prior to loading onto the gel. EMSA supershift/blocking antisera and mAb were either purchased (c-Rel, p65, p52, p50; Santa Cruz Biotechnology, Santa Cruz, CA) or were gifts (p50, p52, Bcl-3; G. Nolan, Stanford University).

DNAse I footprinting

DNAse I footprinting was performed using a derivation of the procedures described by Jones et al. (23) and Durand et al. (22). Binding reactions were carried out under the conditions described above for EMSA but scaled up to 50 μl and performed in protein excess. After binding, using 50 μg nuclear extracts, 50 μl of a 10-mM $MgCl_2$/5 mM $CaCl_2$ solution, and 2 μl of an appropriate DNAse I (Worthington, Freehold, NJ) dilution were added and incubated for 1 min on ice. The amount of DNAse I was optimized for each of the end-labeled probes. Conditions generally ranged from 0.05 μg to 0.2 μg per reaction. DNase I digestion was stopped by adding 90 μl of stop buffer (20 mM EDTA, 1% SDS, and 0.2 M NaCl). After addition of 20 μg yeast transfer RNA as carrier, the samples were extracted two times with an equal volume of phenol/chloroform (1/1) and precipitated after adjusting the solution to 0.3 M sodium acetate and 70% ethanol. DNA samples were resuspended in 4 μl of an 80% formamide-loading dye containing 1× Tris-borate/EDTA electrophoresis buffer, bromphenol blue, and xylene cyanol, heated to 90°C for 2 min, and loaded on 6% polyacrylamide-urea sequencing gels.

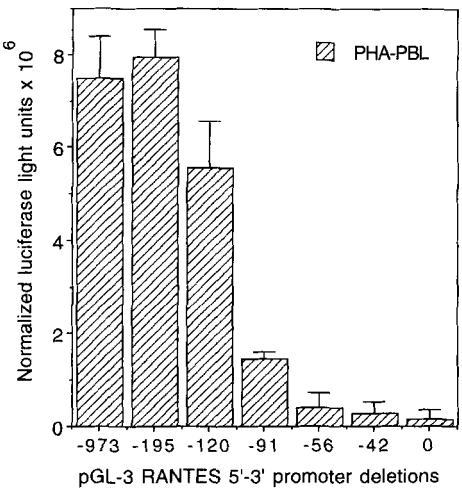

FIGURE 1. A series of 5′ to 3′ deletions of the RANTES promoter were fused to a luciferase reporter gene (*pGL-3*, Promega) and transiently transfected via electroporation into day 2 PHA-activated PBL, then tested for reporter gene activity 36 h later. Results are average values of luciferase activity of triplicates normalized for transfection efficiency and protein content.

Methylation interference

Methylation interference was performed using the protocol of Ausubel et al. and Baldwin and Sharp (24, 25). Briefly, partially methylated single ^{32}P end-labeled probe representing the R(A/B) site (TCGAGCTATTTTG GAAACTCCCCTTAGGGGATGCCCCTCAACTGCTCGA (underlined region corresponds to the DNase I footprint)) site was complexed with nuclear extracts and run out on a nondenaturing 4% polyacrylamide gel as described for EMSA (10-fold scale up). For competition experiments 1000-fold excess of a κB oligomer (TCGAGTCAGAGGGACTTTCC GAGAGCT (underlined sequence denotes the κB consensus)) was added as described above (see EMSA). The resultant gel was not dried, but exposed over 4 h to x-ray film. Regions of the polyacrylamide gel containing bands of various protein/DNA complexes and free oligonucleotide probe were excised, and the modified DNA was removed from the nondenaturing acrylamide gel by electroelution (Electro-Eluter; Bio-Rad, Hercules, CA). Following piperidine organic cleavage (24, 25), the DNA preparations were analyzed on 10% polyacrylamide-urea sequencing gels.

Results

RANTES-luciferase reporter gene fusions define the minimal region required for promoter activity in PHA-activated PBL

The Hut78 cell line is derived from a cutaneous T cell lymphoma (26), and expresses a "late" T cell phenotype (i.e., constitutively expresses IL-2R, IL-2 (15, 26), and RANTES) (16). We previously demonstrated by transient reporter gene assay that approximately 200 nucleotides of the immediate upstream region of the RANTES gene is sufficient for optimal promoter activity in the Hut78 T cell line (14, 16). A similar series of RANTES reporter-gene fusions was used to map the minimal region needed for optimal reporter gene activity in transiently transfected PHA-activated PBL blasts (Fig. 1). PBL were transfected by electroporation 48 h after activation with PHA-P and assayed for luciferase reporter gene activity 36 h later (84 h after activation). Transient transfection of promoter-reporter deletions representing 973, 195, 120, 91, 56, and 42

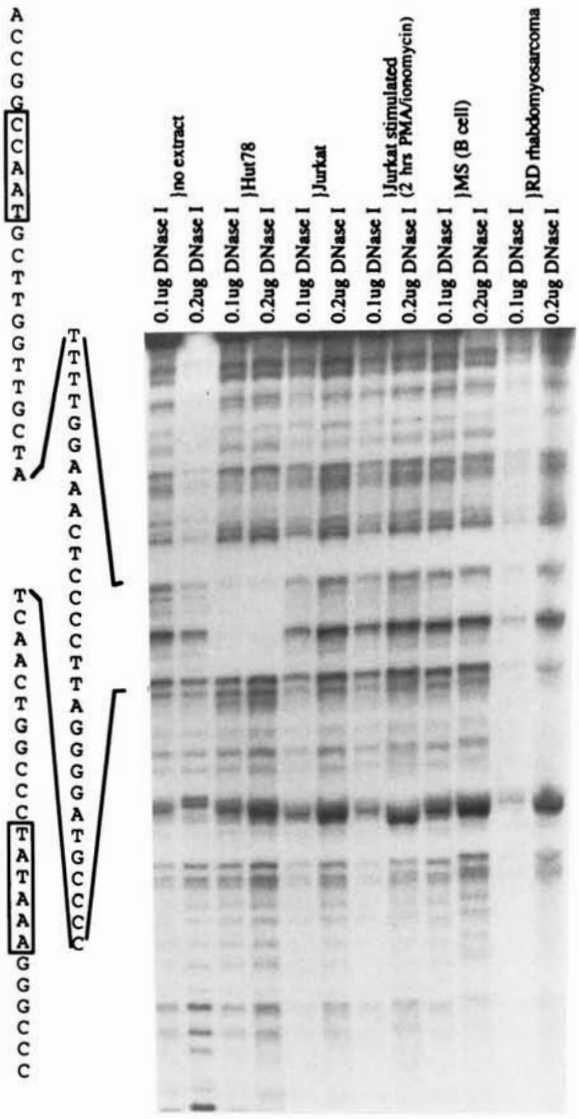

FIGURE 2. Binding of nuclear factors to the immediate 195 nucleotides of the RANTES promoter region and complete 5′ untranslated region was tested by DNase I footprint assay. Nuclear extracts isolated from the T cell line Hut78 protected a region from approximately −42 to −78 from DNase I digestion. Nuclear extracts isolated from Jurkat, activated Jurkat (2 h with PMA (25 ng/ml) plus ionomycin (1 μM)), MS (RANTES nonexpressing cell lines), and RD (a RANTES positive cell line) did not protect this region.

nucleotides upstream of the RANTES site of transcriptional initiation demonstrated that the immediate −195 nucleotides of the RANTES promoter was sufficient for maximal expression of the luciferase reporter in PHA-activated PBL (Fig. 1).

DNase I footprinting of the minimal RANTES promoter identified a large region protected by T cell-derived nuclear extracts

DNase I footprinting may identify potential transcription factor-binding sites. DNase I footprinting using nuclear extracts isolated from Hut78 cells and PHA-activated PBL (day 5) was performed on the *Sac*I *Kpn*I DNA fragment containing the −195 minimal promoter region and the complete 5′ untranslated region of the RANTES gene. A 36-nucleotide region, designated R(A/B), was protected from DNase I digestion in the presence of nuclear extracts isolated from Hut78 (Fig. 2) and day 5 PHA-activated PBL (data not shown). This DNase I footprint, spanning approximately nucleotides −78 to −42, lies between the putative CCAAT and TATAAA boxes (16) (Fig. 2). While this region was protected by nuclear extracts isolated from Hut78 and day 5 PHA-activated PBL, nuclear extracts derived from the Jurkat T cell line, stimulated Jurkat cells (activation with calcium ionophore and PMA for 2 h), MS (Burkitt's B cell lymphoma), and RD (rhabdomyosarcoma cell line) failed to protect this region. Hut78 expresses RANTES constitutively, while MS and Jurkat do not (even after stimulation of the Jurkat cells (27); data not shown). The muscle cell line RD expresses RANTES (1, 16) but did not show an obvious footprint.

The DNA sequence of the DNase I footprint R(A/B): CTATTT TGGAAACTCCCCTTAGGGGATGCCCCTCAA, contains two potential NF-κB-like sites (double and single underline). One site, here designated R(B) (single underline), shows a consensus for a nearly palindromic κB-like binding site (28). The site just upstream, designated R(A) (double underline), also displays κB-like characteristics, especially when analyzed on the opposite DNA strand (GGGAGTTTCC) (28).

EMSA: identification of T cell-expressed nuclear protein complexes that bind to the DNA sequence R(A/B)

EMSA was used to characterize the kinetics and expression of nuclear factors that bind the region designated R(A/B) at different days following the activation of resting peripheral blood T cells. Nuclear extracts isolated from resting PBL, and at days 1, 3, 5, and 7 following activation with PHA, were used in the EMSA experiments. At least four general complexes (labeled bands 1 through 4 in Fig. 3) were found to associate with the R(A/B) oligonucleotide. The nuclear factors comprising band 4 were found in each extract tested. The complexes that yield bands 1, 2, and 3 were induced in PBL following PHA activation. Band 3, which may represent two closely migrating complexes, is seen by day 1 and is variably present in all subsequent time points. The factors responsible for band 2 appear by day 3, result in a broad gel shift band on EMSA, and may comprise several complexes. Band 1 appears last, between days 3 and 5. The same four bands are also found in extracts isolated from a long-term normal $CD8^+$ T cell line (stimulated with alloantigen and conditioned media) (17). It is of interest that bands 1 and 2 temporally correlate with the strong up-regulation of RANTES mRNA expression following alloantigen or PHA activation of resting peripheral blood T cells (3). All four bands, including a very weak band 3, are also found in nuclear extracts isolated from the Hut78 T cell line (not shown).

EMSA competition of the R(A/B) site with a κB consensus oligomer

Because of the two potential κB-like sites found within the R(A/B) region, a κB consensus oligomer (corresponding to the sequence derived from κ light chain promoter: TCGAGTCAGAGG GACTTTCCGAGAGCT) was used in cold oligomer competition with the labeled R(A/B) oligonucleotide probe to determine if the complexes were related to NF-κB (Fig. 4). Nuclear extracts from day 7 PHA-activated PBL were tested with five-fold serial dilutions of cold competing oligomer. At the highest concentration, the cold competitor was approximately 1000-fold in excess over the concentration of labeled oligonucleotide probe. Control competition of the R(A/B) region with itself (Fig. 4) competes all complexes. The κB oligonucleotide efficiently competed for binding to most of band 4 but only partially competed for binding to the other complexes, even at a 1000-fold molar excess of cold competitor. Band 3 was not present in this extract and was generally weak and variably present in nuclear extracts isolated beyond 3 days after

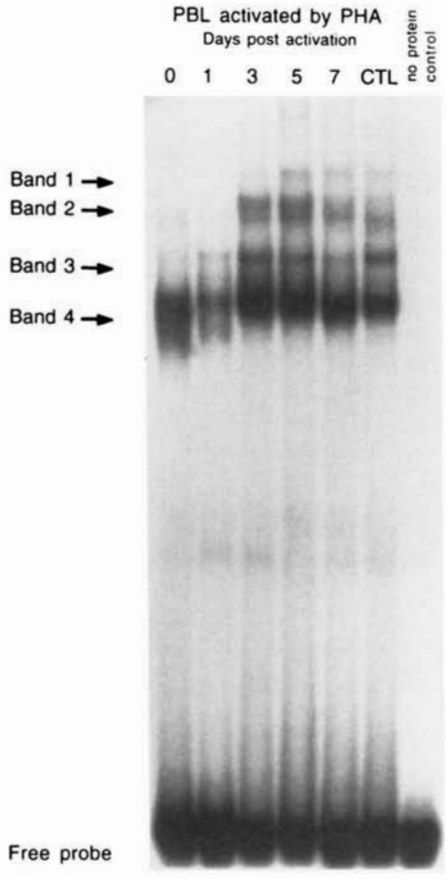

FIGURE 3. EMSA performed using an oligonucleotide probe representing the DNase I footprint identified in Figure 2 (R(A/B): TCGAGC TATTTTGGAAACTCCCCTTAGGGGATGCCCCTCAACTCGA). A series of nuclear extracts derived from resting PBL and from days 1, 3, 5, and 7 after activation with PHA, as well as from an established $CD8^+$ T cell line (CTL) stimulated by alloantigen and conditioned media were used.

PHA activation of PBL. In experiments in which band 3 was evident it was readily competed with cold κB (Ig-κB) oligonucleotide probe (see Fig. 5, *A* and *B*, and the methylation interference experiment detailed below).

Characterization of the R(A/B) DNA sequences required for the observed EMSA patterns

To determine which sequences within R(A/B) allow formation of the various EMSA patterns described above, a panel of oligonucleotide probes representing different regions within the R(A/B) site were used in EMSA with day 5 PHA-activated PBL nuclear extracts (Fig. 5). R(A) (5′ end) and R(B) (3′ end) oligonucleotide probes separate the two putative κB-like sites (Fig. 5*A*). The R(A) oligonucleotide probe gave rise to an EMSA shift that displayed bands 2 through 4, and showed a loss of band 1 (late T cell-derived shift) as compared with the pattern found with the R(A/B) probe. (The apparent diminution of band 2 seen here is due to a slight underloading of this lane.) A truncation of the 5′ end of R(A) (designated R(Am5′)) removed a string of thymidine residues up to the κB-like consensus site (Fig. 5*A*). This change dramatically reduced the efficiency of binding of band 2 (late T cell derived shift), but did not affect binding of bands 3 and 4. A truncation of the cytosine residues on the 3′ end of R(A), designated R(Am3′), eliminated one-half of the κB-like site and completely abolished binding of all complexes. Thus, the binding of the "late" expressed EMSA complex in band 2 is dependent upon the presence of some thymidine residues 5′ to the "classical" κB Rel-binding domain. The requirement for cytosine residues at the 3′ region of the motif indicates that the 3′ κB-like sequences of site R(A) are absolutely necessary for binding of all the complexes to the R(A) site, including the "late" T cell derived factors. Band 1 requires the presence of both the R(A) and R(B) sequences.

The oligomer R(m5′B) extends eight nucleotides 5′ from the κB consensus of R(B); nuclear factor binding to this oligonucleotide probe resulted primarily in band 4. A similar shift was found with the R(B) oligomer which contains only the κB consensus site. The κB control EMSA shift (Ig-κB) displayed two bands, a strong lower band and a faint upper band (Fig. 5*A*).

To determine which of the complexes (bands 1 through 4) depend upon NF-κB, the same experiment was performed in the context of a 1000-fold molar excess of κB (Ig-κB) competing oligonucleotide (right side of Fig. 5*A*). No competition was observed for band 1, while some of band 2 and the majority of band 4 were competed. Thus, the remainder—all of band 1, most of band 2, and part of band 4—are not due to "classical" NF-κB-like binding. This indicates that these bands do not represent any of the known Rel complexes, including p50-p50, p52-p52, p50-p65, p65-p65, and p65-c-Rel (28 and G. Nolan, unpublished observations). The data further demonstrate that the remaining bands (exclusive of band 1) represent binding to the R(A) portion of R(A/B). This includes the band 2 complex up-regulated by day 3 in PHA-activated T cells.

The kinetics of expression of the late T cell-derived, non-NF-κB nuclear factors binding to site R(A/B) termed R(A)FLAT (RANTES site R(A)-derived factors of late activated T cells) are demonstrated in Figure 5*B*. In this experiment, nuclear extracts were again isolated from PBL at various stages following activation with PHA and the subsequent EMSA was performed using labeled R(A/T) oligonucleotide probe in the presence of a 1000-fold molar excess of cold competing κB oligonucleotide. The non-NF-κB proteins are faintly present by day 2, and are still increasing by day 4 following activation. Strong expression is also found in long-term $CD8^+$ CTL lines.

Methylation interference assays determine G residue usage for nuclear factor binding to the R(A/B) site

Methylation interference assays were used to determine the G residues within the R(A/B) site necessary for formation of the complexes generated on EMSA. The results of these experiments, evidenced by the loss of piperidine cleavage product bands, demonstrate two distinct regions of transcription factor binding present in R(A/B) (Fig. 6). The EMSA band 2 formed from nuclear extracts isolated from day 5 PHA-activated PBL depends upon G residues within the R(A) site (GCTATTTTGGAAACTCCCCT TAGGGGATGCCCCTC). Band 3 appears to represent complexes that interact with both R(A) and R(B) sites. Band 4 interacts primarily with G residues in the R(B) region of R(A/B) (GCTATTT TGGAAACTCCCCTTAGGGGATGCCCCTC) (Fig. 6). To distinguish non-Rel-binding characteristics, an excess of cold κB competitor oligomer was added to the nuclear extract prior to incubation with the labeled-methylated probe. Band 3 was completely competed by the cold κB oligonucleotide (not shown). These results corroborate the results of the data detailed in Figure 5; that is, the "late" T cell induced complex forming band 2 and a portion of band 4 are apparently comprised of non-Rel proteins that interact with the R(A) site, and the NF-κB-binding factors interact with both sites.

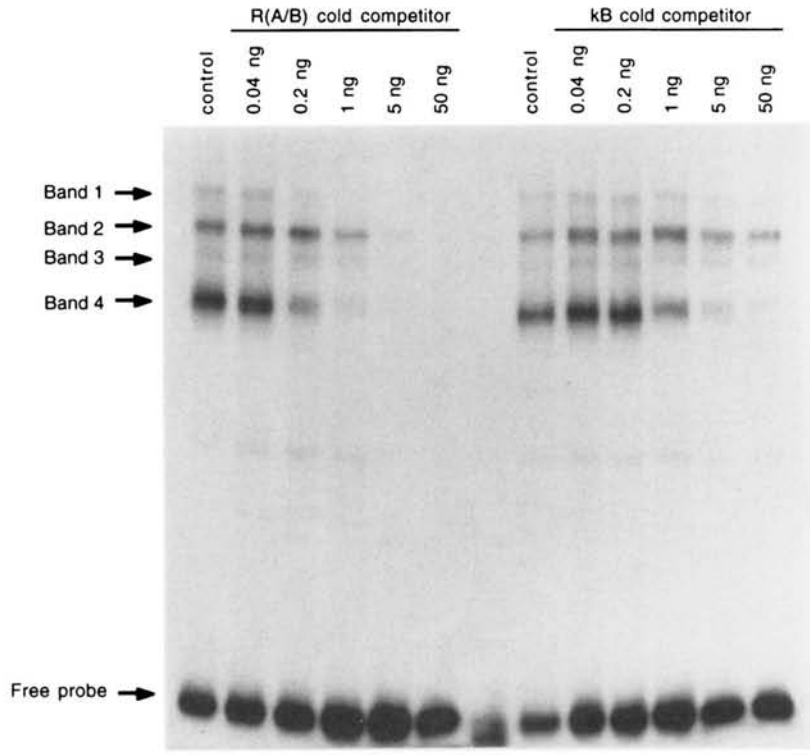

FIGURE 4. EMSA-oligonucleotide competition experiments using day 7 PHA-stimulated PBL nuclear extract were performed using the R(A/B) oligomer as probe. The R(A/B) (as control) and a κB consensus oligonucleotide (lgκB) (TCGAGTCAG AGGGACTTTCCGAGAGCT) were used at increasing concentrations as competitors.

Supershift EMSA using anti-Rel family-specific mAbs and antisera defines Rel components that bind the R(A/B) region

A series of supershift antisera specific for the Rel family proteins, p50, p52, Bcl-3, and mAbs to c-Rel and p65, were used in EMSA to determine which Rel factors bind the κB-like regions within R(A/B). Controls for NF-κB binding were performed using nuclear extracts isolated from 4-h TNF-α-stimulated dermal fibroblasts (Fig. 7*A*). TNF-α is a potent inducer of NF-κB (Rel p50-p65 heterodimers) in dermal fibroblasts (29). The TNF-α-stimulated fibroblast control EMSA results showed one major band with a minor band migrating slightly faster in the gel formed with the R(A/B), R(A), and κB oligonucleotide probes. The R(B) showed two bands present at approximately equal intensity (Fig. 7*A*). The major complex in R(A/B), R(A), and κB, and the upper band seen with R(B), were competed for or shifted by both the anti-p50 antisera and the anti-p65 mAb, suggesting that this complex represents Rel p50-p65 heterodimer. The lower complex was shifted by the anti-p50 antisera in each of the oligonucleotide probes tested, but was unaffected by the anti-p65 mAb (Fig. 7*A*). Similarly, other Rel family-specific Ab reagents, including mAbs to p52, Bcl-3, and c-Rel, had no effect on either complex (data not shown), indicating that the lower complex represents Rel p50-p50 homodimers. These results indicate that the R(A/B) region contains two sites with the capacity to bind Rel proteins. Site R(A) appears to bind Rel p50-p65 heterodimers at a high efficiency and Rel p50-p50 homodimers less well, while site R(B) strongly binds Rel p50-p50 homodimers but also binds Rel p65-p50 heterodimers with less efficiency. Finally, the late T cell-derived complex was not present in the TNF-α-activated fibroblast extracts.

The experiment was then repeated using extracts from day 5 PHA-stimulated PBL (Fig. 7*B*). Only a very weak band 3 was seen in these experiments. The κB control EMSA shift showed one strong band and a minor band, which ran slightly slower on the gel. The band 4 complex formed on R(A/B), R(A), and R(B) probes and the major κB complex, was supershifted by the anti-p50 antisera, while the R(A/B)-, R(A)-, and R(B)-derived EMSA shifts were unaffected by the p65 mAb. The apparent diminution of signal intensity in band 4 (lane R(B)-p65) was not seen in repeated experiments and is probably due to slight underloading of probe or protein in that lane. The upper portion of the κB control shift was slightly altered by the anti-p65 Ab, which may represent the presence of some p50-p65 in these extracts. The anti-p50 antisera and anti-p65 mAb had no effect on the late T cell-derived bands 1 or 2 formed on R(A/B) or band 2 generated with R(A). The other Rel-specific antisera and mAbs (antisera or mAbs to p52, Bcl-3, or two different mAbs to c-Rel) did not affect the EMSA patterns seen with the R(A/B) probe (data not shown). These results suggest that most of band 4, the portion that is competed by κB oligonucleotides (Figs. 4–6), comprises p50-p50 homodimers.

As described earlier, the complex that forms band 3 is highly variable in different PBL nuclear extracts, especially in preparations isolated after day 3 following PHA activation of PBL. In instances in which it could be studied in T cell extracts, the band was readily competed by κB oligonucleotides (Figs. 5 and 6). To formally identify the proteins contained in band 3, EMSA supershift assays were performed using nuclear extracts from 40-h PHA-activated PBL. These assays showed detectable levels of band 3, and low but detectable amounts of band 2 (Figs. 3 and 5*B*). In these experiments, the formation of band 3 was blocked by the anti-p65 Ab and the anti-p50 antisera, suggesting that band 3 is formed in part by Rel p65-p50 heterodimer (Fig. 7*C*).

Site-specific mutagenesis of the R(A), R(B), and R(A/B) sites demonstrates that these sites are required for RANTES promoter-reporter gene activity in T cells

To address the functional role of sites R(A), R(B), and R(A/B) in control of RANTES expression in T cells, deletions of these regions were made in the −195 "minimal promoter" RANTES-luciferase reporter gene construct (Fig. 1). In each of these mutants,

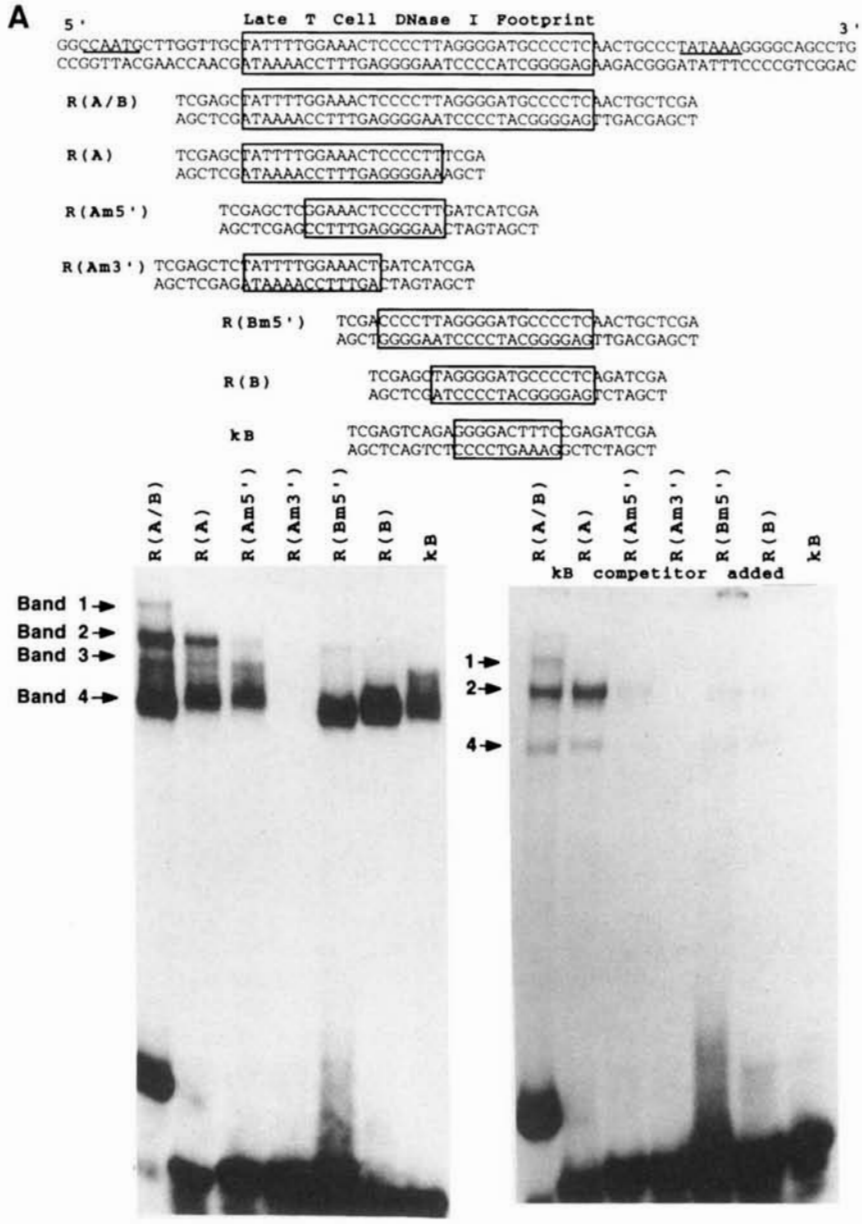

FIGURE 5. *A*, A series of truncated oligonucleotides derived from the R(A/B) region were used to map the binding patterns seen on EMSA in day 5 PHA stimulated PBL nuclear extract. The *right side* of the autoradiogram represents a parallel experiment performed in the presence of a 1000-fold molar excess of cold κB (lg-κB) competing oligonucleotide. *B*, The kinetics of expression of the non-NF-κB-derived factors binding to the oligonucleotide R(A/B), termed R(A)FLAT, was determined from PHA-activated PBL, using nuclear extracts derived from resting PBL from days 1, 2, 3, and 4 after PHA activation, and from an established $CD8^+$ T cell line (CTL). The EMSA were performed in the presence of a 1000-fold molar excess of the cold κB (lgκB) oligonucleotide.

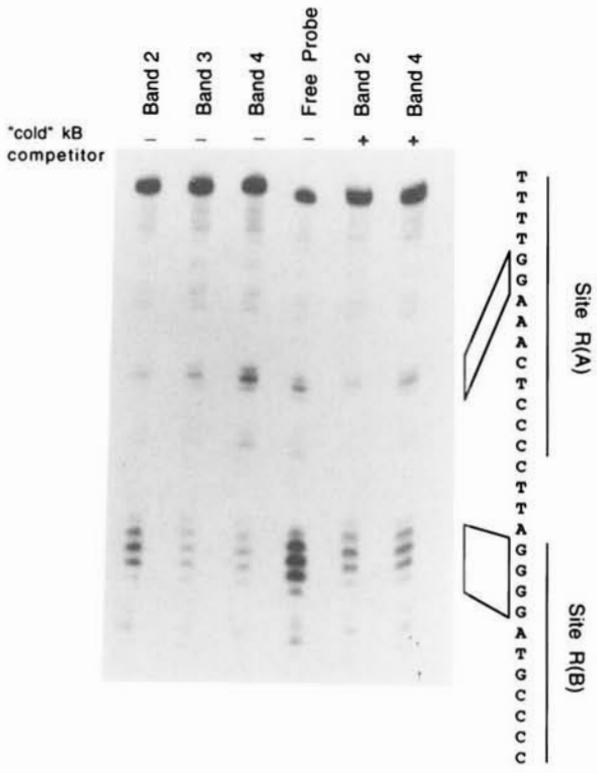

FIGURE 6. Methylation interference was used to determine the G residue usage in binding to the R(A/B) region. Bands 2, 3, and 4 were isolated as described (25) and subjected to piperidine degradation. Bands 2, 4 were also isolated in the presence of a 1000-fold molar excess of cold κB competing oligomer and subjected to degradation. Band 3 was completely competed by the addition of cold κB competing oligonucleotide.

the site of interest was replaced with approximately the same number of non-sense nucleotides to preserve spatial arrangement of the sites within the promoter (Fig. 8, *A* and *B*). The mutant constructs were transiently co-transfected with RSV-β galactosidase control reporter gene plasmids into PHA-activated PBL and Hut78 cells and subsequently tested for reporter gene activity (Fig. 8*B*). Elimination of site R(A/B) from the pGL-3 RANTES (−195) construct resulted in loss of over 90% of promoter activity in both the PHA-stimulated PBL and in Hut78 cells. Deletion of site R(A) similarly resulted in loss of at least 90% of the promoter activity in both cell types. Elimination of site R(B) resulted in the variable loss of between 40% and 75% of promoter reporter gene activity in both PHA-activated PBL or Hut78 cells depending upon the individual experiment.

The R(A/B) region was also tested in enhancer activity assays. Following its cloning as a trimer in the 5′-3′ orientation, into the pGL-2 Pro vector (Stratagene, San Diego, CA), it was tested in transient transfection in promoter-reporter gene assays. In transfection-controlled experiments, this construct consistently demonstrated a 6- to 10-fold enhancement over the pGL-2 Pro vector alone in PBL and Hut78 cells (not shown).

Discussion

In contrast to the detailed understanding of the transcriptional controls of T cell immediate early genes such as IL-2, little is understood about what regulates expression of genes induced at the later stages of peripheral T cell development (days 3 to 5) (15, 30). The RANTES chemokine gene is strongly up-regulated at 3 to 5 days after T cell activation (3, 15) and thus provides an opportunity to investigate the transcriptional controls operating during this stage of T cell development. We previously described the RANTES cDNA (3), its genomic organization (16), putative promoter region (16), and two regulatory sites within the RANTES promoter functionally important for expression in T lymphocytes (14). Here we report analysis of an additional control region that transcriptionally regulates the RANTES chemokine gene in mitogen-activated peripheral blood T lymphocytes and the T cell tumor line Hut78.

DNase I footprinting analysis of the RANTES promoter defined a region from approximately −78 to −42, termed site R(A/B), which was protected by nuclear extracts isolated from activated T cells. Electrophoretic mobility shift assays using a series of oligonucleotide probes derived from the protected region, competing consensus oligonucleotides, methylation interference assays, and a panel of anti-transcription factor mAbs identify two Rel/κB consensus-binding regions within the R(A/B) region. Site R(B) binds p50-p50 homodimers preferentially and p50-p65 heterodimers less efficiently, while site R(A) has a high affinity for Rel p50-p65 heterodimers and also binds p50-p50 homodimers to a lesser degree. Site R(A/B) also binds a group of potentially novel nuclear factors that are specifically up-regulated 3 to 5 days after the activation of resting T cells and are expressed in functionally mature $CD8^+$ CTL.

NF-κB is an inducible heterodimeric complex composed of 50-kDa and 65-kDa subunits (28). Cellular activation leads to the nuclear translocation of the NF-κB complex and the regulation of a host of genes (28). Since Rel p50 homodimers are present constitutively in the T cell nucleus while p65-p50 heterodimers are induced transiently early in T cell activation (31), these proteins are not likely themselves to direct the late up-regulation of RANTES gene expression in PBL. The Rel heterodimers p50-p65 do appear to play a role in the immediate early induction of RANTES in fibroblasts and epithelial cells (J. M. Pattison, manuscript in preparation).

The kinetics of the non-NF-κB R(A/B)-binding proteins described here are consistent with the temporal regulation of RANTES mRNA expression in T cells (3). EMSA indicates that the factors which complex to form bands 1 and 2 are up-regulated in PHA-activated PBL, with maximal expression from days 3 to 5, coincident with the up-regulation of RANTES mRNA (3) (Fig. 3). The binding region for the complex that forms band 2 includes DNA sequences outside the recognized Rel/κB consensus (28). The complexes forming bands 1 and 2 are not competable with κB oligonucleotides and do not react with Ab reagents to known Rel family members. Sites R(A) and R(B) both appear critical for the functional activity of the RANTES promoter in activated T lymphocytes, as specific mutations of either site result in loss of 40 to 90% of the transcriptional activity as evidenced by transient reporter gene assays. We hypothesize that the factors here termed R(A)FLAT (RANTES site R(A)-derived factors of late activated T cells), either alone or in combination with Rel proteins, temporally regulate RANTES mRNA expression through the R(A/B) region in normal T cells. Recent DNase I footprint assays carried out in nuclear protein excess suggest that binding of R(A)FLAT to site R(A) may be stabilized through interaction with Rel p50-p50 bound at site R(B) (P. J. Nelson, unpublished data).

NFATc is a distant member of the Rel family (32–34) and members of the C/EBP interact with Rel proteins (32). The complexes forming band 1 and band 2 of R(A)FLAT could not be competed with consensus oligonucleotide sequences that bind human NFAT (33), C/EBP (NFIL-6) (35), Ets-1 (36), AP-1 (22), or Oct-1 (37) (data not shown). The R(A/B) derived complexes that form bands

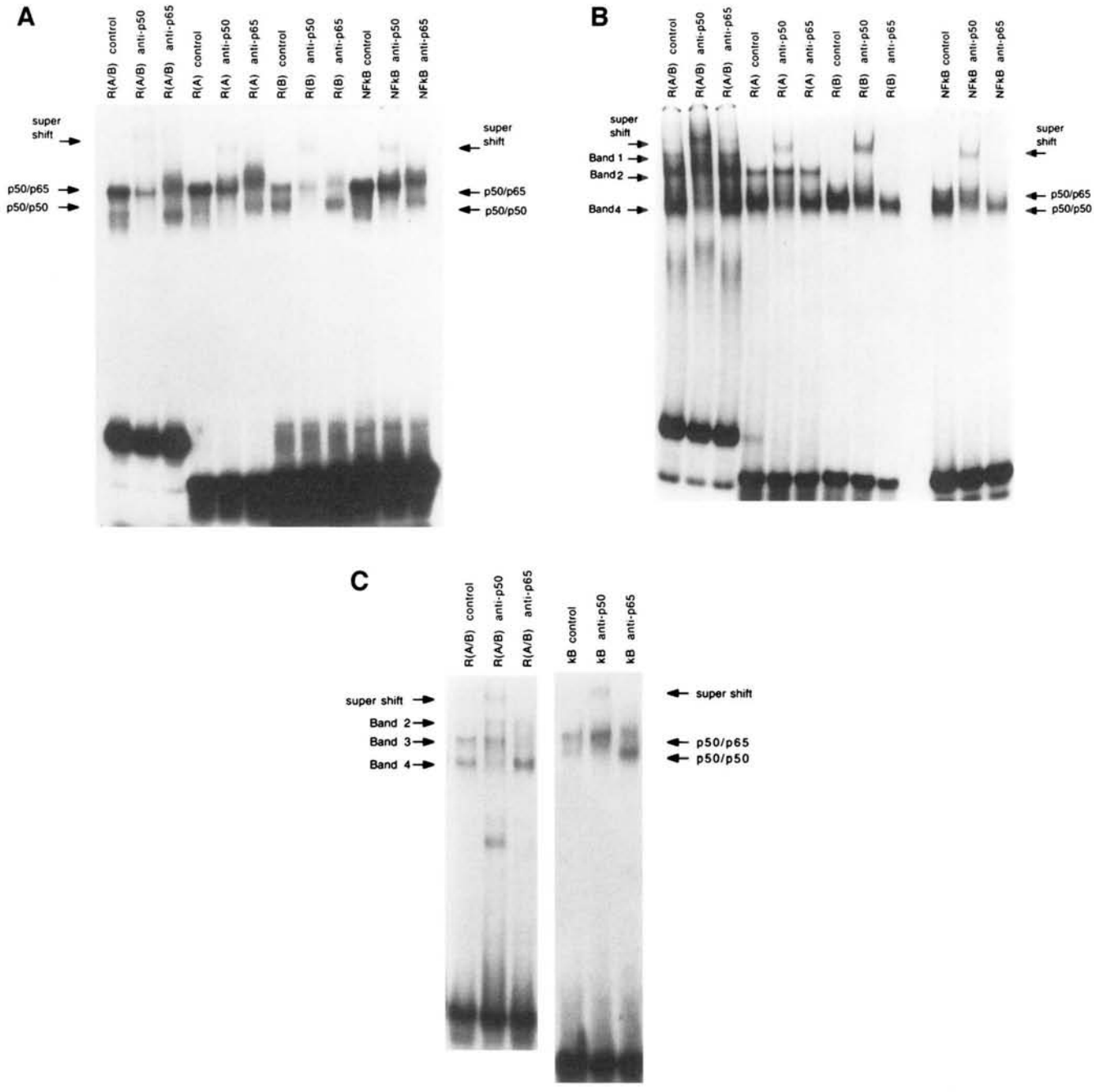

FIGURE 7. *A*, EMSA supershift experiments were performed using anti-p50 antisera and anti-p65 mAb reagents, control nuclear extracts isolated from TNF-α-stimulated (4-h) fibroblasts and labeled R(A/B), R(A), R(B), and κB oligonucleotide probes. Sites R(A) and R(B) bind both Rel p50-p65 heterodimers and p50-p50 homodimers. The anti-p50 antisera supershifted, while the anti-p65 mAb primarily blocked specific binding. *B*, The experiment was repeated using day 5 PHA-activated PBL nuclear extracts. *C*, In general, day 5 to day 7 PHA-stimulated PBL nuclear extracts were found to have low levels of band 3. However, nuclear extracts isolated 40 h after stimulation of PBL with PHA show some band 2, and detectable levels of bands 3 and 4. Supershift EMSA assay demonstrates that band 3 represents Rel p50-p65. (All of these experiments were performed in the presence of protease inhibitors.)

1 and 2 could not be supershifted or competed with Abs or antisera that recognize NFATc or Ets family factors (data not shown).

Independently binding transcription factor components of disparate families can interact to affect transcriptional activation through novel sequences (32, 34, 35, 38). For example, Rel family members interact with non-Rel proteins (NFIL-6) to regulate the IL-8 promoter (35). The high mobility group protein I(Y) (HMG I(Y)) interacts with the minor groove of the A-T-rich region at the center of the κB sequence within the human IFN-β promoter (38). Furthermore, distant Rel family members, such as the cytoplasmic component of NF-AT, interact with leucine zipper proteins, such as Fos and Jun, to act on κB-like sequences (32, 34). It is possible that an analogous interaction occurs at the R(A) site between Rel family members and the novel late factors identified by EMSA to induce RANTES message in T cells.

What is not clear is the extent to which new transcriptional initiation per se contributes to the overall up-regulation of RANTES mRNA late in T cell activation. Indeed, the existence of post-transcriptional controls for RANTES mRNA accumulation may also be important to the late up-regulation of RANTES mRNA in the rapidly proliferating functionally mature T lymphocytes.

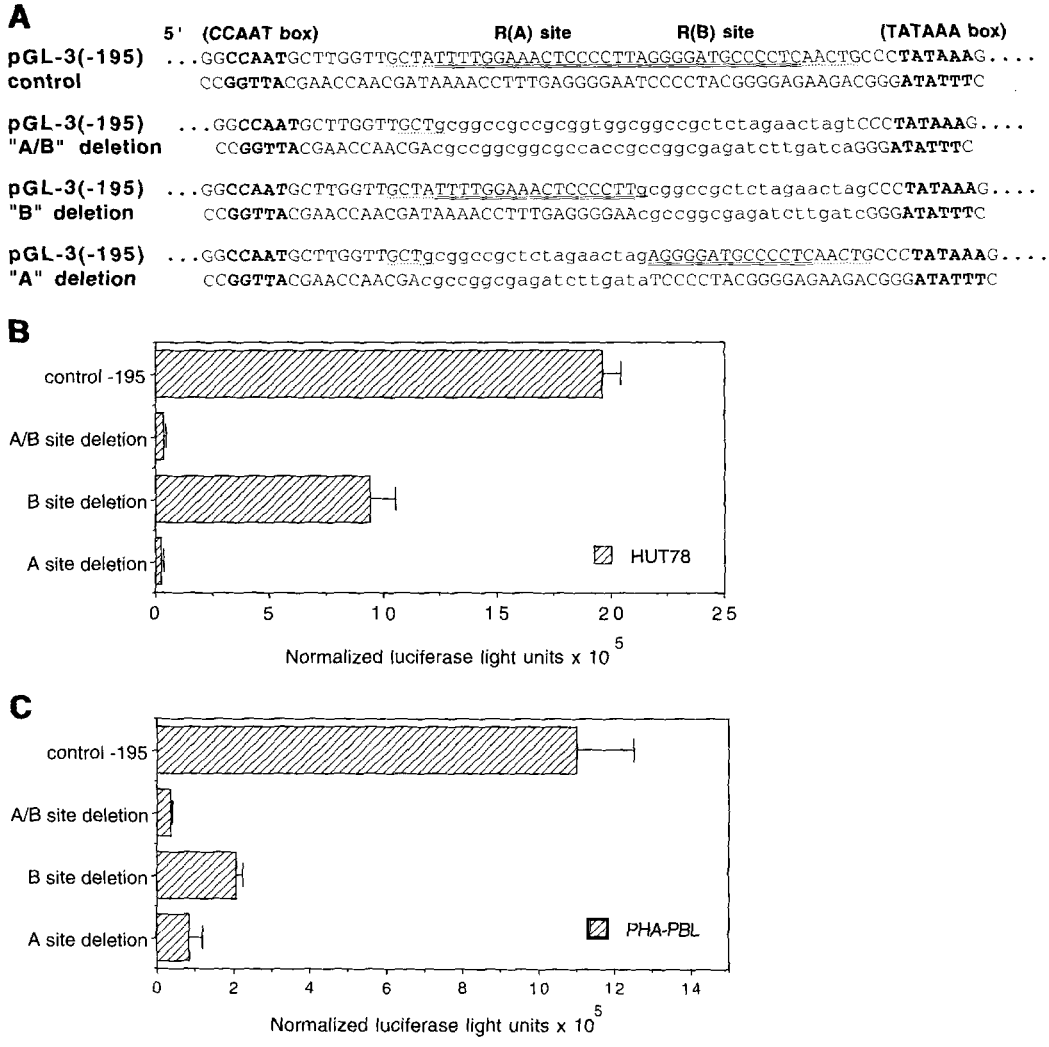

FIGURE 8. *A*, Site-specific mutations that eliminated site R(A/B), R(A), and R(B) were generated using PCR. Lower case font represents the substituted nucleotides. These constructs were used in transient reporter gene assays using the Hut78 cell line (*B*) and PHA-activated PBL (*C*) (electroporated 48 h after PHA activation and assayed 36 h later). Results are averages of quadruplicates for Hut78 and triplicates for PBL normalized for transfection efficiency and concentration of protein extract. A representative experiment is shown in each figure.

Understanding the transcriptional regulation of the RANTES chemokine may prove important in a variety of diseases. Inhibition of RANTES expression may be therapeutic in diseases characterized by cellular infiltration (39). Alternatively, the up-regulation of RANTES expression may prove useful for the therapy of AIDS (4). Lastly, the characterization of novel "late" expressed transcription factors may provide insight into T cell maturation.

Acknowledgments

We thank Dr. Garry P. Nolan (Department of Pharmacology, Stanford University) for the gift of various anti-Rel family antisera and for input into the experiments described here. We also thank Drs. Gerald R. Crabtree and Garry P. Nolan (Stanford University) for critical reading of this manuscript.

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