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# Negative transcriptional regulation in anergic T cells

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**ABSTRACT** **Anergy is a mechanism of T-lymphocyte tolerance induced by antigen-receptor stimulation in the absence of costimulation, whereby T cells exhibit a defect in antigen-induced transcription of the interleukin 2 (IL-2) gene. Here we present evidence for a mechanism of negative IL-2 gene regulation in anergic T cells. High amounts of binding activity to the negative regulatory element A (NRE-A) of the IL-2 promotor were detected in nuclear extracts from human T cells shortly after induction of anergy. Rapid induction of this nuclear complex is blocked by cyclosporin A and is found to be independent of protein synthesis. Plasmid DNAs, containing either the human phorbol 12-myristate 13-acetate-responsive element (PRE) or both NRE-A and PRE, were used as template for *in vitro* transcription assays in the presence of T-cell nuclear extracts. Under these conditions nuclear extracts from both anergic and rested T-cell clones, after crosslinking of CD3 and CD28, induced transcription of plasmids containing only PRE. However, when plasmids containing NRE-A and PRE were used, transcription was only induced by nuclear extracts from rested but not anergic T cells. These findings suggest the functional relevance of transcriptional repression of the IL-2 gene in anergic T cells.**

Tumor-specific antigens can be demonstrated on many neoplasms by immunization and challenge experiments, but they do not normally elicit a sufficiently strong immune response to prevent tumor growth in immunocompetent hosts (1). Studies designed to gain a better understanding of this phenomenon demonstrated that efficient activation of T cells requires costimulation of the CD28 receptor (1–4). Inadequate costimulation of tumor-reactive T cells may contribute to the fact that antigenic tumors are not normally rejected by the immune system (1, 5). We recently reported that a $CD4^+$ T-cell clone and an autologous major histocompatibility complex class $II^+$ melanoma cell line interact with each other, which leads to an increase of intracellular $[Ca^{2+}]$ in the T-cell clone (6). This interaction failed to induce interleukin 2 (IL-2) production or proliferation of the T-cell clone but rendered it unresponsive to subsequent stimulation.

As shown previously, anergic T cells have a defect in antigen-induced transcription of the IL-2-encoding gene (7). Molecular characterization of this defect suggests a block in transcription at the level of the transactivation factor AP-1 as a cause for T-cell-clonal anergy (8), although alterations in tyrosine phosphorylation after T-cell-receptor occupancy in the absence of costimulation have also been reported (9). However, the exact mechanism for the anergy phenomenon still remains to be defined.

In this report we extend the characterization of molecular events during induction and maintenance of anergy using the model system described above. High amounts of binding activity to the negative regulatory element A (NRE-A) of the IL-2 promotor were detected in nuclear extracts from human T cells shortly after induction of anergy. Functional transcription assays revealed that this binding activity could inhibit the transactivational activity of AP-1 in stimulated, anergic T cells. These findings suggest a possible mechanism for negative transcriptional regulation of IL-2 in anergic T cells.

## MATERIAL AND METHODS

**Melanoma Cell Lines.** Cells were dispersed by grating tumor fragments from freshly resected melanomas against a 0.3 × 0.3 mm metallic mesh immersed in complete medium (CM), consisting of RPMI 1640 medium/5% fetal calf serum/1% penicillin/1% streptomycin/1% glutamine/1% sodium pyruvate. These dispersed cells were then plated into six-well collagen-coated culture plates at 2000 cells per well in CM and grown in humidified incubators at 37°C with 5% $CO_2$. Tumor cell lines were maintained as monolayers and passaged by trypsination as required.

**Generation of Cloned Human Tumor Infiltrating Lymphocyte Lines.** Cell suspensions were prepared by mincing freshly resected tumors into small pieces, digesting them for 12 hr in CM/0.1% collagenase type IV (200 units/g)/0.005% deoxyribonuclease type I (100 units/mg) (Sigma), and then passing these cells through a steel sieve. After washing twice with Hanks' balanced salt solution (HBSS), viable mononuclear cells were separated on a Ficoll/Hypaque gradient at 600 × *g* for 20 min and suspended in culture medium. These cells were then used for cloning by limiting dilution—i.e., plating 0.5 cell per well onto a feeder layer. Each well consisted of 5000 autologous, irradiated (50 Gy) melanoma cells obtained from the same tumor specimen, and 5000 irradiated (50 Gy) cells of the Epstein–Barr virus-transformed B-cell line LAZ509. Colonies were expanded by the addition of culture medium containing IL-2 (200 international units/ml) every 3 days. This culture medium consisted of RPMI 1640 medium/20% human AB serum/1% penicillin–streptomycin/1% glutamine/1% sodium pyruvate. All cell lines used in this study were subcloned at least three times.

**RNA Isolation and PCR Amplification.** Cells ($10^6$) were lysed in 0.5 ml of guanidinium buffer (4 M guanidinium isothiocyanate/50 mM Tris·HCl/10 mM EDTA/2% sarcosyl/100 mM 2-mercaptoethanol). After phenol/chloroform extraction, the lysate was layered onto a 5.7 M CsCl gradient and centrifuged at 50,000 rpm at 20°C for 3 hr in a TLS-55 rotor (Beckman). The RNA pellet was resuspended in the presence of 20 units of RNase inhibitor (Boehringer Mannheim) and treated with 10 units of RNase-free DNase I (Promega) for 30 min at 37°C to eliminate remaining genomic DNA. Synthesis of cDNA was done by incubating a reaction mixture (20 μl) for 60 min at 37°C. This mixture contained the following: reverse

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Abbreviations: EMSA, electrophoretic mobility-shift assay; IL-2, interleukin 2; NRE-A, negative regulatory element A; RC, responsive complex; CD28RE, CD28 responsive element; PRE, phorbol 12-myristate 13-acetate; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; CM, complete medium.

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transcriptase reaction buffer (Pharmacia), 0.5 mM deoxynucleotide triphosphate, 20 units of RNase inhibitor, 50 pM oligodeoxythymidylate, and 20 units of Moloney murine leukemia virus reverse transcriptase (Pharmacia). cDNA was heated to 95°C and then chilled on ice. A 2.5-μl sample of the cDNA mixture was used in a 50-μl PCR reaction mixture. This reaction mixture contained 10 mM Tris·HCl, 50 mM KCl, 0.01% gelatin, 2.0 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 2.5 units of *Taq* polymerase (Promega), and 1 μM sense and antisense oligonucleotide primer. Samples were amplified in a DNA thermocycler (Pharmacia) for 35 cycles. Each cycle consisted of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min. An aliquot of the PCR product was then subjected to electrophoresis on a 6% polyacrylamide gel and visualized by staining with ethidium bromide. The following primers were used: Nil-2-a A, (sense, 5′-TAAAGCACTATGACCTAAAGC-3′); Nil-2-a B, (antisense, 5′-TAACCCTGTGTATTTCTGGAT-3′); β-actin-A, (sense, 5′-GAGGGAAATCGTGCGTGACATCAA-3′); and β-actin-B, (antisense, 5′-GGAACCGCTCGTTGCCAATAGTGA-3′).

**Electrophoretic Mobility-Shift Assay (EMSA).** Nuclear extracts were isolated from $10^7$ cloned T cells by the method of Schreiber *et al.* (10). Cells were suspended in 400 μl of cold buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol/0.5 mM phenylmethylsulfonyl fluoride). The cells were then allowed to swell on ice for 15 min, after which 25 μl of a 10% (wt/vol) solution of Nonidet P-40 was added, and the tube contents were vigorously mixed. After centrifugation the nuclear pellet was resuspended in 80 μl of ice-cold buffer C (20 mM Hepes, pH 7.9/0.4 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride) and vigorously rocked at 4°C for 15 min. Nuclear extracts were cleared by centrifugation. Protein content was assayed with Bradford reagent (Bio-Rad). $^{32}$P-End-labeled double-stranded oligonucleotides containing the following human recognition sequences were used (11, 12): NF-AT, GATCGGAGGAAAAACTGTTTCATGGAGGAAAAACTGTTTCAT; octamer, GATCATGCAAATGATCATGCAAATGATCATGCAAAT; NF-κB, GATCCAAGGGGACTTTTCCATGGATCCAAGGGGACTTTCCATG; AP-1, GATCCTTCGTGACTCAGCGGGATCCTTCGTGACTCAGCGG; CD28 responsive complex (RC), AAAGAAATTCCAAAGAAAAGAAATTCCAAAGA; NRE-A, AGCTTCAGACAGGTAAAGTCAGACAGGTAAAGT.

Each reaction mixture contained 10 fmol of probe and 5 μg of protein from nuclear extracts. Binding reactions were conducted in 50 mM Hepes/30% (vol/vol) glycerol/50 mM KCl/10 mM MgCl/1 mM EDTA/1 mM dithiothreitol and 1 μg of poly(dI-dC) in a final volume of 15 μl. These reactions were allowed to occur at 4°C for 20 min. Complexes were resolved on 6% nondenaturing polyacrylamide gels in 50 mM Tris, 45 mM borate/0.5 mM EDTA, pH 7.4. The samples were subjected to electrophoresis for 2 hr at 10 V/cm. Gels were dried and autoradiographed at −70°C overnight.

**Templates for *in Vitro* Transcription.** The chloramphenicol acetyltransferase (CAT) reporter plasmids pBL-CAT2 and p1xPRE-CAT containing either the thymidine kinase (TK) promotor alone or together with an AP-1 binding site have been described (13). Oligonucleotides that contained two copies of the NRE-A site (CAGACAGGTAAAGATCAGACAGGTAAAGA) were ligated into the *Sph* I/*Sal* I sites of p1xPRE-CAT upstream of the AP-1-binding site to form pNRE-PRE-CAT. A second construct prepared in a similar fashion (mNRE-PRE-CAT) was identical to pNRE-PRE-CAT, except for the underlined T → C point mutation (CAGACAGGCAAAGATCAGACAGGCAAAGA). These plasmids were prepared under standard conditions and were linearized by digestion with *Sma* I. After digestion, the templates were extracted once with phenol and twice with chloroform and then precipitated with ethanol/LiCl and resuspended in $H_2O$ at 50 ng/μl.

***In Vitro* Transcription Assays.** Nuclear extracts were prepared from T cells by the method of Dignam *et al.* (14). Transcription was done in a volume of 25 μl under the following conditions: 500 ng of linearized template DNA, 100 μg of nuclear extract, 12 mM Hepes, pH 7.9/12% (vol/vol) glycerol/0.3 mM dithiothreitol/0.12 mM EDTA/60 mM KCl/12 mM $MgCl_2$/600 μM each of ATP, CTP, and UTP, and 60 μM [α-$^{32}$P]GTP (10 Ci/mmol; 1 Ci = 37 GBq). Transcription complexes were preassembled in the absence of nucleotides for 30 min at 30°C. After preassembly, nucleotides were added from a 5× stock solution, and transcription was allowed to proceed for 30 min at 30°C. *In vitro* transcription products were purified and analyzed on 7 M urea/6% polyacrylamide sequencing gels and subsequently visualized with a Molecular Dynamics PhosphorImager.

**Transfection.** Human B7 cDNA subcloned into the pBJ expression vector was provided by Lewis Lanier (DNAX). Melanoma cells were transfected with 5 μg of pBJ/B7 plasmid by electroporation (280 V, 960 μF) with a Gene Pulser (Bio-Rad) and selected in CM supplemented with G418 at 1 mg/ml (GIBCO). Transfectants were maintained in CM/G418 at 0.5 mg/ml.

## RESULTS AND DISCUSSION

The human CD4$^+$ T-cell clone STC3 was generated from tumor-infiltrating lymphocytes of a major histocompatibility

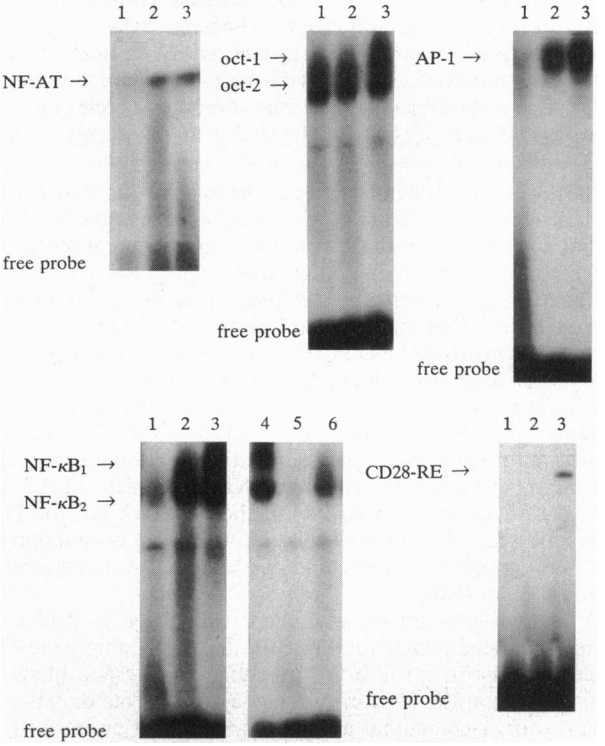


FIG. 1. Comparison of induced DNA-binding complexes in anergic and activated helper T cells. Nuclear extracts were isolated from $10^7$ cloned T cells without stimulation (lanes 1); 4 hr after interaction with wild type (lanes 2), or B7-transfected melanoma cells (lanes 3). $^{32}$P-End-labeled double-stranded oligonucleotides containing human NF-AT, octamer, NF-κB, AP-1, or CD28RC recognition sequences were used. Each reaction contained 10 fmol of probe and 5 μg of protein of nuclear extracts. In the case of the NF-κB recognition sequence, binding reactions of nuclear extracts isolated from $10^7$ cloned T cells 4 hr after interaction with B7-transfected melanoma (lanes 3 and 4) were also done in the presence of 50-fold molar excess of unlabeled NF-κB (lane 5) and CD28RC (lane 6) recognition sequences. A representative of three experiments is shown.

complex class II-positive melanoma (6). Interaction of this T-cell clone with the autologous melanoma cell line rendered it anergic to subsequent stimulation. We reported recently that transfection of this melanoma cell line with the costimulatory molecule B7 converted it into a fully competent antigen-presenting cell, inducing IL-2 production by and proliferation of clone STC3. Here we used the same model system to compare the induction of nuclear factors that regulate IL-2 transcription in anergic and activated T cells. Specifically, nuclear extracts were isolated from STC3 cells 4 hr after interaction with wild-type or B7-transfected melanoma cells. These extracts were then analyzed by gel-mobility shift assays for the presence of NF-AT1, octamer, NFκB, AP-1, and CD28 RC (Fig. 1). Interaction with wild-type melanoma cells induced a profile of these transcription factors that differed from that induced by interaction with B7 transfectants; neither $NF\kappa B_1$ nor the CD28 RC was induced after engagement of the T-cell receptor in the absence of accessory signals. Because members of the NFκB family such as c-Rel, p50, and p65 were recently shown to contribute to the formation of the CD28 RC (15), we performed a specific competition of the DNA–protein complex with unlabeled oligonucleotide containing the CD28 responsive element (CD28RE) and consensus κB oligonucleotide. As shown in Fig. 1, the CD28RE oligonucleotide could compete with $NF\kappa B_1$.

T-cell clonal anergy is characterized by a failure to produce IL-2 upon subsequent restimulation, even in the presence of costimulatory signals. Therefore, we compared the induction of NF-AT1, octamer, NFκB, AP-1, and CD28RC in either rested or anergic T cells 3 hr after crosslinking of CD3 and CD28. For this purpose STC3 cells were rendered anergic by an 8-hr coculture with the autologous melanoma cell line 72 hr before restimulation. This comparison revealed no significant differences detectable by EMSA in the amounts of these transcription factors present in nuclear extracts obtained from either rested or anergic T cells after restimulation (data not shown). This result suggests an additional mechanism for inhibition of IL-2 production in anergic T cells because upon optimal stimulation, both anergic and rested cells expressed a substantial set of activating DNA-binding activities that would otherwise be sufficient to induce IL-2 gene transcription.

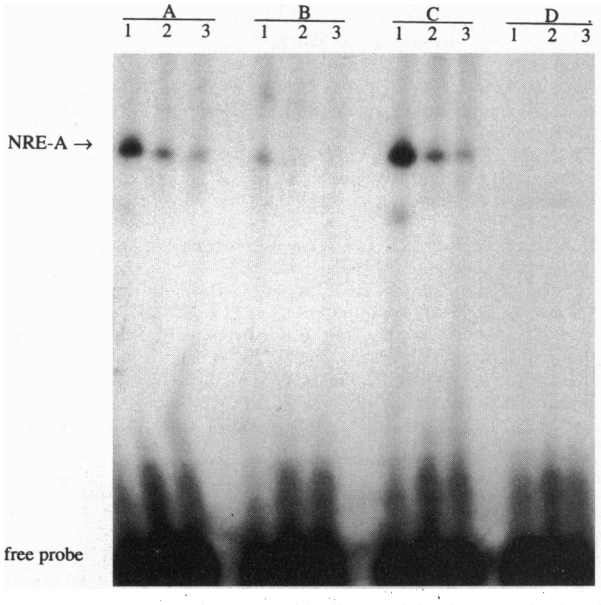


FIG. 2. Induction of NRE-A-binding activity in anergic T cells. Nuclear extracts were isolated from $10^7$ cloned T cells, 4 hr after interaction with the wild-type (*A*) or B7-transfected melanoma cells (*B*) after interaction with wild-type melanoma cells in the presence of 10 μM cycloheximide (*C*) or 1 μM cyclosporin A (*D*). Each reaction contained 10 fmol of $^{32}$P-end-labeled double-stranded oligonucleotides representing human NRE-A, 5 μg of protein of nuclear extracts (lanes 1), and unlabeled NRE-A (5-fold or 50-fold molar excess; lanes 2 and 3, respectively). Binding reactions were conducted as described above. A representative of three experiments is shown.

Kang *et al.* (8) demonstrated that transactivation mediated by AP-1 is affected by anergy. However, as shown here, as well as by others (16), the molecular profiles of AP-1-binding proteins in nuclear extracts from anergic or activated T cells do not differ in gel mobility-shift assays. Recently, a nucleotide sequence element (NRE-A) within the IL-2 gene was identified that negatively regulates IL-2 expression (12). NRE-A is recognized by the nuclear factor Nil-2-a, which is able to entirely abolish the phorbol 12-myristate 13-acetate-induced activity of a PRE-NRE-A CAT construct. Therefore, induction of Nil-2-a would explain the impaired transactivation by AP-1 in anergic T cells. To test this hypothesis, nuclear extracts were isolated from STC3 cells 4 hr after interaction with wild-type or B7-transfected melanoma cells and analyzed by EMSA for the presence of NRE-A-binding factors. Stimulation by wild-type melanoma cells induced a higher NRE-A-binding activity than stimulation with the B7 transfectants (Fig. 2). The induction of this nuclear complex was independent of protein synthesis but was blocked by cyclosporin A. In this regard it should be mentioned that the induction of clonal anergy can also be blocked by cyclosporin A (17). Kinetic studies revealed that the NRE-A-binding activity could be detected up to 48 hr after interaction of STC3 with wild-type melanoma cells. When protein synthesis is blocked, this binding activity could still be detected, but only up to 12 hr after coculture (Fig. 3). This result suggests that some NRE-A-binding activity exists in resting T cells. Because the sustained presence of the NRE-A-binding factor requires protein synthesis, we used reverse transcription-PCR to determine the induction of Nil-2-a mRNA by interaction of STC3 cells with the wild-type melanoma cells (Fig. 4). Indeed, Nil-2-a mRNA could be detected from 4 to 24 hr after induction of anergy. It should be noted that induction of anergy could possibly be inhibited by blocking of protein synthesis (18). Taken together, these findings indicate that both induction of the *nil-2-a* gene and posttranslational modification of preexisting proteins that contribute to the NRE-A-binding activity are involved in the induction and maintenance of T-cell anergy.

To confirm the functional relevance of the NRE-A-binding activity detected in nuclear extracts of anergic T cells, we introduced a tandem repeat of the NRE-A sequence into the p1xPRE-CAT plasmid that contained a binding site for AP-1 upstream of the TK promotor linked to CAT. Because functional transcriptional assays in cloned T cells are usually hampered by the low and various transfection efficiencies of reporter constructs into these cells, we performed a series of *in vitro* transcription assays with nuclear extracts prepared from either rested or anergic cloned T cells 3 hr after crosslinking of CD3 and CD28 by the method of Dignam *et al.*

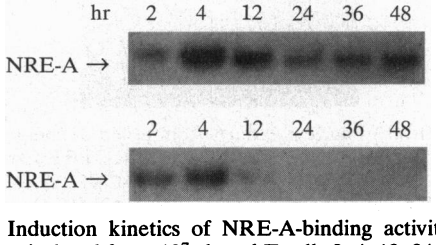


FIG. 3. Induction kinetics of NRE-A-binding activity. Nuclear extracts were isolated from $10^7$ cloned T cells 2, 4, 12, 24, 36, and 48 hr after interaction with wild-type melanoma in medium alone (*Upper*) or in the presence of 10 μM cycloheximide (*Lower*). Each reaction contained 10 fmol of $^{32}$P-end-labeled double-stranded oligonucleotides representing human NRE-A and 5 μg of protein of nuclear extracts. Binding reactions were conducted as described above. A representative of three experiments is shown.

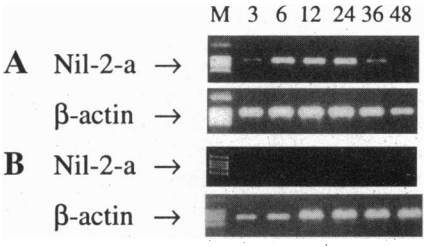


FIG. 4. Kinetics of Nil-2-a mRNA induction. RNA obtained from $10^6$ cells of the T-cell clone STC3 3, 6, 12, 24, 36, and 48 hr after interaction with the wild-type (*A*) or B7-transfected melanoma (*B*) was analyzed by reverse transcription and PCR amplification with primers Nil-2-a (*A*) and Nil-2-a (*B*) as described. The cDNAs were normalized to the β-actin PCR product. An aliquot of PCR product was electrophoresed on a 6% polyacrylamide gel and visualized by ethidium bromide staining. A representative of three experiments is shown.

(14). The anergic cells were obtained by an 8-hr coculture of STC3 cells with the autologous melanoma cell line 72 hr before restimulation. When plxPRE-CAT was used as template DNA, we detected no difference in the transcription efficiency induced by nuclear extracts obtained from rested or anergic T cells (Fig. 5). However, when pNRE-PRE-CAT was used as template, only nuclear extracts obtained from rested T cells induced a significant amount of *in vitro* transcription. The amount of transcription from pNRE-PRE-CAT induced by nuclear extracts obtained from anergic T cells was as low as the

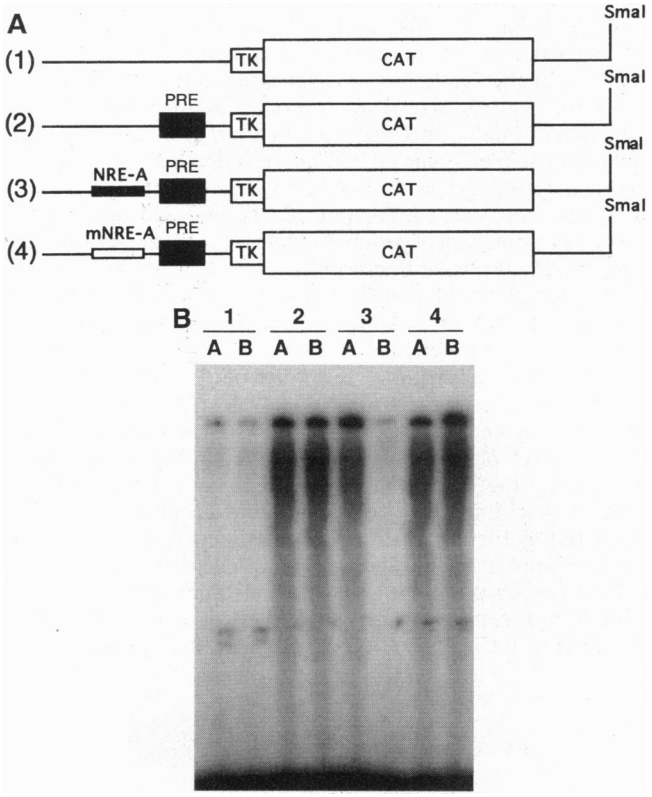


FIG. 5. Effects of nuclear extracts from rested or anergic T cells on *in vitro* transcription of plxPRE-CAT and pNRE-PRE-CAT. (*A*) Diagram of CAT reporter constructs used: (1), pBL-CAT, (2), plxPRE-CAT, (3), pNRE-PRE-CAT, (4), and mNRE-PRE-CAT. (*B*) Nuclear extracts were prepared from $10^7$ either rested (lanes A) or anergic (lanes B) cloned T cells 3 hr after crosslinking of CD3 and CD28. Anergy was induced by an 8-hr coculture with the autologous melanoma cell line 72 hr before restimulation. Transcription reactions were done by incubation of 500 ng of linearized pBL-CAT (*1*), plxPRE-CAT (*2*), pNRE-PRE-CAT (*3*), and mNRE-PRE-CAT (*4*) with 100 μg of nuclear extracts as described. A representative of three experiments is shown.

basic transcription from pBL-CAT. This reporter construct contains the TK promotor but no AP-1-binding site. For these experiments a reporter construct containing a mutated NRE-A and a normal PRE site upstream of the TK promotor served as the control. This construct gave no differences in the amount of *in vitro* transcription when nuclear extracts obtained from either rested or anergic STC3 cells were used. This finding confirms the specific inhibition of transcription mediated by these NRE-A-binding proteins.

Schwartz *et al.* (7, 19, 20) proposed a model for the induction of an anergy factor whenever helper T type 1 cells are stimulated through their T-cell receptor, regardless of the presence or absence of a costimulatory signal. This inhibitory factor is proposed to be diluted out by proliferation of fully activated T cells but not in those cells that are rendered anergic. Our finding of a NRE-A-binding factor in fully activated STC3 cells is consistent with this hypothesis (Fig. 2). However, we also observed a significant difference in the amount of NRE-A-binding complex induced in anergic versus activated T cells. Therefore, it seems likely that under physiological conditions the negative regulatory transcription factor Nil-2-a is produced in small amounts to terminate antigen-induced IL-2 transcription, but it is produced in larger quantities once T cells are rendered anergic.

In summary, we propose a model where the interplay between inhibiting factors such as NRE-A-binding activity and activating factors such as the CD28 RC regulate the state of helper T type 1 cell activity. Although, studies on the regulation of transcription often focus on mechanisms of transcriptional activation, our results point out that transcriptional repression may also be an important factor in the regulation of genes.

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