MOLECULAR AND CELLULAR BIOLOGY, Mar. 1997, p. 1314–1323 Vol. 17, No. 3
0270-7306/97/$04.00+0

Costimulation by B7-1 and LFA-3 Targets Distinct Nuclear Factors That Bind to the Interleukin-2 Promoter: B7-1 Negatively Regulates LFA-3-Induced NF-AT DNA Binding

EDUARDO PARRA,[1] MIKAEL VARGA,[2] GUNNAR HEDLUND,[1,2] TERJE KALLAND,[1,2] AND MIKAEL DOHLSTEN[1,2]*

Wallenberg Laboratory, Section for Tumour Immunology, Department of Cell and Molecular Biology, University of Lund,[1] and Pharmacia & Upjohn,[2] Lund, Sweden

Received 16 April 1996/Returned for modification 10 July 1996/Accepted 27 November 1996

We have characterized the regulation of nuclear factors involved in transcriptional control of the interleukin-2 (IL-2) promoter-enhancer activity in Jurkat T cells stimulated with superantigen presented on HLA-DR transfectants combined with the ligands LFA-3 (CD58) and B7-1 (CD80). Gel shift analyses showed that NF-AT was strongly induced in LFA-3-costimulated Jurkat T cells, suggesting that NF-AT is a key target nuclear factor for the CD2–LFA-3 pathway. Studies using HLA-DR–B7-1–LFA-3 triple transfectants showed that the LFA-3-induced NF-AT DNA binding activity was negatively regulated by B7-1 costimulation. In contrast, induction of a CD28 response complex containing only c-Rel proteins was seen after B7-1 costimulation. Both LFA-3 costimulation and B7-1 costimulation induced the AP-1 and NF-κB nuclear factors. Distinct compositions of the NF-AT complexes were seen in B7-1- and LFA-3-costimulated cells. LFA-3 induced primarily Jun-D, Fra-1, and Fra-2, while B7-1 induced June-D–Fos complexes. In contrast, AP-1 and NF-κB complexes induced in B7-1- and LFA-3-costimulated T cells showed similar contents. Transient transfection of Jurkat T cells with a construct encoding the IL-2 enhancer-promoter region (position −500 to +60) linked to a luciferase reporter gene revealed that B7-1 costimulation was required to induce strong transcriptional activity. Combined B7-1–LFA-3 costimulation resulted in a synergistic increase in IL-2 transcriptional activity. Multimers of the AP-1, NF-AT, NF-κB, and CD28 response elements showed distinct kinetics and activity after LFA-3 and B7-1 costimulation and revealed that B7-1 and LFA-3 converge to superinduce transcriptional activity of the AP-1, NF-AT, and CD28 response elements. Transcriptional studies with an IL-2 enhancer-promoter carrying a mutation in the CD28 response element site revealed that the activity was reduced by 80% after B7-1 and B7-1–LFA-3 costimulation whereas the transcriptional activity induced by LFA-3 was unaffected. Our data strongly suggest a selectivity in induction of nuclear factors by the CD2–LFA-3 and CD28–B7-1 pathways. This selectivity may contribute to regulation of the levels of IL-2 induced by LFA-3 and B7-1 costimulation and favor autocrine and paracrine T-cell responses, respectively.

T lymphocytes require at least two signals in order to be activated. The first signal involves ligation of the T-cell receptor (TCR) by specific peptide-major histocompatibility complexes, and a second signal is provided via costimulatory adhesion ligand-receptor pair interactions (30–32, 48). CD2 (15) and CD28 (55) are the two major adhesive receptors which are present on a large fraction of T cells and they provide costimulatory signals after interaction with the ligands LFA-3 and B7-1 (1, 4, 33, 35, 43, 59). The CD2–LFA-3 pathway has previously been shown to contribute to antigen-independent T-cell adhesion, to induce a large amount of IFN and TNF, but to induce only low levels of interleukin-2 (IL-2). The CD28–B7-1 pathway superinduces IL-2 production and supports paracrine cell growth (43, 50). It has further been demonstrated that ligation of the CD28 receptor can prevent the induction of anergy in T cells (24), while the CD2–LFA-3 pathway may have a role in the reversal of an established anergic state (6). Thus, a dynamic interplay between the CD28 and CD2 pathways seems to have a central role in regulating the balance between activation and anergy. Moreover, evidence for cross-talking between the CD2 and CD28 pathways to increase the production of IL-2 and other cytokines has been reported (33, 43). IL-2 is the major T-cell growth factor which is transcribed in resting T cells after ligation of the TCR combined with adequate costimulation. The critical regulatory and inducible activity of the IL-2 gene enhancer-promoter region occurs within the 300-bp region immediately upstream of the start site (14, 21, 44). A number of nuclear elements in the IL-2 gene promoter have been shown to be involved in the regulation of IL-2 gene transcriptional activity (14, 21, 44). These include the distal binding site of the nuclear factor of activated T cells (NF-AT) (52), the nuclear factor κB (NF-κB) binding site (51), the activation protein 1 (AP-1)-responsive element (2, 37), the CD28-responsive element (CD28RE) (18), and the octamer (OCT-1)-responsive element (34). However, the physiological extracellular signals that trigger the activation of the nuclear factors that bind and activate these response elements in T cells are poorly defined. The NF-κB/Rel family of transcription factors includes p50 (NFKB1), p52 (NFKB2), p65 (RelA), RelB, and c-Rel (22, 23). In the inactive state, members of the NF-κB/Rel family are in complex with specific inhibitory subunits called IκB proteins (3, 25, 39). At least six species (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105), which associate with NF-κB/Rel proteins, are known (5).

The most well-characterized IκB protein is IκBα (54). Cell activation induces the phosphorylation of IκBα and its subsequent proteolytic degradation (8, 54). The NF-κB complex dissociates from the negative regulator IκB, translocates to the

* Corresponding author. Mailing address: Dept. of Cell and Molecular Biology, Lund University, Box 7031, S-220 07 Lund, Sweden. Phone: 46 46 2229253. Fax: 46 46 2224201.

nucleus, and binds to the decameric sequence motif (25, 39). The CD28RE within the IL-2 promoter contains a sequence similar but not identical to the sequence of the κB site. Several studies have demonstrated that members of the NF-κB/Rel family of proteins are involved in the nuclear protein complex that binds to the CD28RE, suggesting that this response element is a modified κB response motif (19, 36, 58). The CD28RE was originally defined as a major and unique response element for the CD28–B7-1 signal transduction pathway (18). However, recent data suggest that induction of binding of nuclear proteins to the CD28RE is not exclusive to the CD28 pathway (12). The AP-1 transcription factor is a complex composed of proteins belonging to the Jun and Fos families. Heterodimers formed by one member of each family display higher DNA binding capacity and more effective transactivating properties than homodimers formed by members of the Jun family alone. The consensus AP-1 binding site has been identified in several genes involved in T-cell activation (2, 37). In addition, the AP-1 protein was demonstrated to complex with both NF-AT and OCT-1, suggesting that this factor may act in concert with other transcription factors to regulate a variety of response elements (10, 13, 26, 27). NF-AT was originally described as a cyclosporine (CsA)-sensitive factor binding to the IL-2 promoter in T cells (7, 40, 45). At least two related but distinct NF-AT proteins, NF-ATp and NF-ATc, seem to exist in T cells. These proteins have identical binding specificities and activation requirements (41, 42). Recent studies have demonstrated that NF-ATp is transcriptionally active in B lymphocytes (11, 57) and that activated NF-AT may contain members of the Fos and Jun families (26, 27, 47), indicating that NF-AT is a multicomplex protein.

We have recently shown that costimulation of T cells with B7-1 and with LFA-3 generates distinct cytokine profiles (43). Moreover, the CD2–LFA-3 and CD28–B7-1 pathways seem to converge to superinduce T-cell cytokines (43). In the present study the molecular base for CD2- and CD28-mediated costimulation of T cells has been analyzed at the transcriptional level. We demonstrate that the CD2 and CD28 pathways induce distinct patterns of nuclear factors. The NF-AT protein was found to be a potential target nuclear factor for the CD2–LFA-3 pathway, while the induction of a prominent c-Rel protein containing the CD28RE binding complex was solely induced by the CD28–B7-1 pathway. This strongly suggests a selectivity in the induction of nuclear factors by the CD2 and CD28 pathways. Moreover, the CD2 and CD28 pathways converged to superinduce NF-AT-responsive-element, AP-1-responsive-element-, and CD28RE-driven transcription in T cells.

MATERIALS AND METHODS

Reagents. Staphylococcal enterotoxin E (SEE) was purchased from Toxin Technology (Madison, Wis.). The protease inhibitors phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, and Bestatin were purchased from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). [γ-^{32}P]ATP was purchased from Amersham International (Amersham, England). T4 polynucleotide kinase and poly(dI-dC)$_2$ were obtained from Pharmacia Biotech Inc. (Piscataway, N.J.). Tris-borate-EDTA buffer and acrylamide-bisacrylamide, 29:1, were purchased from Bio-Rad Laboratories (Upplands Väsby, Sweden). Gel supershifts were performed with polyclonal antibodies (Abs) against c-Jun, Jun-B, Jun-D, c-Fos, Fra-1, Fra-2, p50, p65, and c-Rel purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

Transfected cell lines. Chinese hamster ovary (CHO) cells were transfected with cDNA encoding the HLA-DR4, B7-1, and LFA-3 cell surface molecules, as described in detail elsewhere (43, 20). The cell transfectants were generously provided by D. Sansom, Bath, United Kingdom. The different CHO cells used in this study were CHO (untransfected), CHO-DR (singly transfected to express the HLA-DR molecule), CHO-DR/B7-1 (doubly transfected to express both HLA-DR and human B7-1 molecules on their surfaces), CHO-DR/LFA-3 (doubly transfected to express both HLA-DR and human LFA-3 molecules on their surfaces), and CHO-DR/B7-1/LFA-3 (triply transfected to express HLA-DR, human B7-1, and human LFA-3 molecules on their surfaces). Single, double, and triple transfectants expressing similar levels of the transfected molecules were obtained by repeated cell sortings. The surface expression of HLA-DR, LFA-3, and B7-1 was confirmed regularly by fluorescence-activated cell sorter analysis.

Cell culture. The human Jurkat leukemia T-cell line was maintained in culture in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal calf serum (complete medium). All tests on the Jurkat T cells were performed at a concentration of 10^6/ml in complete medium. The transfected CHO cells were irradiated (8,000 rads) and added to the Jurkat cells at a concentration of 0.1×10^6/ml (16).

Plasmid construction. The human IL-2 promoter-enhancer fragment (−500 to +60) was subcloned from plasmid SV-IL-2-CAT into the luciferase vector pGL-2 (Promega, Madison, Wis.). The −500-to-+60 fragment was prepared by PCR with primers that created a 5′ *Xho*I site and a 3′ *Hin*dIII site. These sites were used for overhang ligation into pGL-2 to create pIL-2-luciferase (pGL-2-Luc). The IL-2 promoter mutated in the CD28RE region was generated by PCR-directed splicing overlap extension. The PCR replaced 6 bp within the CD28RE (from −159 to −164) in the IL-2 enhancer of the pGL-2-IL-2-Luc vector with the sequence 5′-CCTCGA-3′. The final plasmid was sequenced to verify that no unwanted mutations were introduced in the process of cloning. The AP-1–luciferase reporter plasmid driven by the rat prolactin minimal promoter (RPP) (−36 to +37) under the control of four copies of the human AP-1 site (46) was kindly provided by M. Rincón and R. A. Flavell (Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Conn.). In order to produce reporter plasmids containing multimers of the recognition sites for NF-κB, NF-AT, and the CD28 response complex (CD28RC), double-stranded oligonucleotides containing these sites as well as appropriate restriction enzyme sites were synthesized, cleaved, and ligated into the *Xho*I site of the pLuc-prolactin minimal promoter plasmid. The orientation for each element was confirmed by restriction enzyme cleavage. The tandem sequences used to construct the different multimer-containing plasmids were as follows: (i) four copies of the CD28RE (5′-AAAGAAAATTCC-3′), (ii) three copies of the distal NF-AT binding site of the human IL-2 promoter (5′-GGAGAAAAACTGTTTCATACAGAAGGC-3′), (iii) four copies of the AP-1-responsive element (the 12-*O*-tetradecanoylphorbol-13-acetate [TPA]-responsive element of the human collagenase promoter) (5′-TCGATTGAGTCAGGGTAA-3′), and (iv) two copies of the NF-κB binding site of the human Ig κ light chain enhancer (5′-GGGACTTTCC-3′).

DNA transfection and luciferase activity analysis. Transfection of Jurkat cells was carried out by electroporation. Briefly, plasmid DNA was mixed with exponentially growing Jurkat cells (20×10^6 cells/ml) in complete medium, and the cells were electroporated in an electrocell manipulator 600 (BTX, San Diego, Calif.) at 130 V and a capacitance of 1,700 μF. The transfected cells were cultured for 24 h before being stimulated with the different CHO transfected cell lines with or without 100 ng of SEE/ml. After various periods of time, cells from each independent well were harvested, washed twice in phosphate-buffered saline (PBS), and treated with lysis buffer (luciferase assay; Promega) for 5 to 10 min on ice. Lysates were spun down for 1 min, and all of the supernatants were analyzed with luciferase reagent (Promega) and measured as duplicates in a luminometer (MicroLumat LB 96 P; Berthold) for 5 s. The background measurement was substracted from the measurement for each duplicate. Experimental values are expressed either as recorded light units of luciferase activity or as relative activity compared to the activity of extracts from unstimulated cells.

IL-2 bioassay. The amount of IL-2 in supernatants was determined in a microassay based on IL-2-dependent proliferation of the CTLL murine cell line as described previously (43). Human Jurkat T cells (10^6/ml) were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well microtiter plates (0.2 ml/well) with the different CHO transfectants (10^5 cells/ml) in either the absence or presence of SEE (100 ng/ml). Cultured supernatants were collected after 72 h of incubation and analyzed for IL-2 content. Supernatants (50 μl) from 72-h cultures were mixed with 5×10^3 IL-2-dependent CTLL cells in 50 μl of medium (R10IF plus β-mercaptoethanol) and incubated for 21 h. Cells were pulsed with 0.5 μCi of [^{3}H]thymidine and harvested 4 h later.

Preparation of nuclear extracts. Human Jurkat leukemia T cells (3×10^7 to 5×10^7) were stimulated with various irradiated transfected CHO cells (8,000 rads) in the absence or presence of SEE (100 ng/ml) at 37°C (in a humidified atmosphere containing 5% CO_2). The cells were harvested after 6 h of culture, and T cells were separated from CHO cells by a plastic adherence technique. The harvested T cells were resuspended in 10 ml of PBS and pelleted by centrifugation for 5 min at 1,500 × *g*. The pellet was resuspended in 1 ml of PBS, transferred into an Eppendorf tube, and repelleted by spinning for 15 s in a microcentrifuge. The PBS was removed, and the cell pellet was resuspended in 500 μl of cold buffer A (10 mM HEPES, pH 7.8; 15 mM KCl; 2 mM $MgCl_2$; 0.1 mM EDTA; 1 mM dithiothreitol; 1 mM PMSF). T cells were allowed to swell on ice for 15 min, 25 μl of a 10% solution of Nonidet P-40 was added, and the tube was vortexed vigorously for 10 s. The homogenate was centrifuged, the nuclear pellet was resuspended in 100 μl of ice-cold buffer B (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM dithiothreitol; 1 mM PMSF), and the tube was rocked for 15 min at 4°C on a shaking platform. The nuclear extract was centrifuged for 5 min at 4°C and the supernatant was frozen in aliquots at −70°C. Before use of buffers A and C, a mixture of the protease

inhibitors was added as follows: 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin/ml, 1 μg of aprotinin/ml, and 40 μg of Bestatin/ml. Usually, 1 to 2 μl of the nuclear extract (2 to 4 μg of protein) was used for a gel shift assay in the presence of 3 μg of poly(dI-dC) as described by Parra et al. (44) and Schreiber et al. (49).

Gel mobility shift assay (gel shift). The double-stranded oligonucleotides used in the mobility shift assay were the OCT-1 consensus, 5′-CGTCTCATGCGAT GCAAATCACTTGAGATC-3′; AP-1 consensus, 5′-CTAGTGATGAGTCAG CCGGATC-3′; NF-κB consensus, 5′-GATCGAGGGGACTTTCCCTAGC-3′; CD28RE site of the human IL-2 promoter, 5′-CTCAAGATCAGAAATTCCA AAGAGAC-3′; the distal NF-AT site of the human IL-2 promoter, 5′-GGAG AAAAACTGTTTCATACAGAAGGC-3′; and the distal NF-AT site of the murine IL-2 promoter, 5′-GATCGCCCAAAGAGGAAAATTTGTTTCATAC AG-3′. The probes were 5′ end labeled with [γ-^{32}P]ATP as single DNA strands by using T4 polynucleotide kinase (Promega) and then hybridized with the corresponding strands and purified on 5% polyacrylamide gels in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) (44). Nuclear protein extracts (3 to 5 μg/μl) were added to binding buffer (4 μl) containing 2 to 3 μg of poly(dI-dC)$_2$ as a nonspecific competitor. The reaction mixtures were incubated at 37°C for 30 min with 15,000 cpm of double-stranded ^{32}P-labeled oligonucleotides in a final volume of 15 μl. The samples were electrophoresed on 5% polyacrylamide gels in 1× TBE. The gels were fixed (40% methanol and 10% acetic acid) for 15 min, dried for 30 to 40 min at 80°C, and autoradiographed. For supershift analysis, the mixtures of nuclear extract were incubated with 1 to 2 μg of Abs against various transcription factors for 20 min before addition of ^{32}P-labeled oligonucleotides. The blocking or shifting effect of Abs against Rel family proteins on band shifts obtained with NF-κB-responsive element or CD28RE probes was tested. The blocking or supershift of Abs against Fos and Jun proteins on band shifts with AP-1- or NF-AT-responsive element probes was also tested. No effect was observed in gel shift assays with control IgG or irrelevant monoclonal Ab. Control experiments performed with the various Abs and DNA probes in the absence of protein extract demonstrated that none of the Abs used bound directly to the DNA target sequences (data not shown).

RESULTS

B7-1 and LFA-3 costimulate IL-2 protein secretion and IL-2 gene promoter transcriptional activity. We used a model which mimicks the natural exposure of ligands to T cells by presentation of bacterial superantigens on CHO cells transfected with human major histocompatibility complex class II (HLA-DR), B7-1, LFA-3, or their combinations. Recent studies of murine T-cell clones have indicated that CD28-mediated superinduction of IL-2 is only marginally related to transcriptional regulation (56). To elucidate the role of the CD2 and CD28 pathways in IL-2 secretion and transcription, we correlated the release of IL-2 to transcriptional activity by an IL-2 promoter-reporter gene assay. A large amount of IL-2 was produced by B7-1-costimulated Jurkat cells, an increase in the amount of IL-2 was induced by CHO-DR/B7-1/LFA-3 triple transfectants, and a low level of induction was observed in LFA-3-costimulated T cells (Fig. 1A). Jurkat T cells transiently transfected with the human IL-2 enhancer-promoter region fused to a luciferase reporter gene showed a 40-fold increase in luciferase activity after B7-1 costimulation (Fig. 1B). In contrast, LFA-3-costimulated cells showed only a four- to fivefold increase in luciferase activity (Fig. 1B). B7-1–LFA-3 costimulation augmented the transcriptional activity to about 80 times the level seen in unstimulated cells, suggesting a cooperative effect on T cells when the CD2 and CD28 receptors were ligated (Fig. 1B). Jurkat T cells stimulated with SEE–HLA-DR in the absence of costimulation showed marginal IL-2 secretion and IL-2 promoter transcriptional activity.

LFA-3 and B7-1 costimulation induces distinct nuclear factors in Jurkat T cells. Transcription of the IL-2 promoter-enhancer is controlled by a number of nuclear factors, including NF-κB, AP-1, NF-AT, the CD28RC, and OCT-1. In order to characterize the pattern of nuclear factors specifically induced after LFA-3 and B7-1 costimulation, we performed electrophoretic mobility shift assays (EMSA) with nuclear extracts prepared from costimulated Jurkat T cells and oligonucleotides encoding the CD28RE and the NF-κB, AP-1, human NF-AT (hNF-AT), murine NF-AT (mNF-AT), and OCT response elements (Fig. 2 and 3). Unstimulated Jurkat T cells did not contain detectable levels of nuclear factors binding to the CD28RE or to the NF-κB or AP-1 response elements (Fig. 2). Similar results were obtained with oligonucleotides encoding the hNF-AT and mNF-AT response elements (Fig. 3A and B). However, all the samples contained large amounts of OCT response element binding proteins (Fig. 3C).

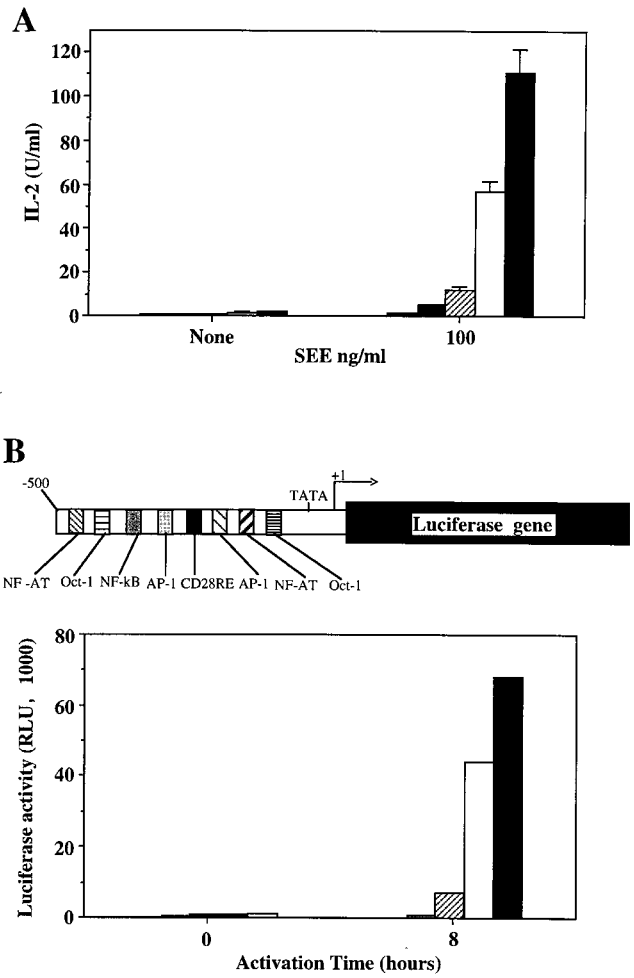

FIG. 1. Correlation between IL-2 production (A) and transcriptional activity (B) in Jurkat T cells. (A) Jurkat T cells (10^6/ml) were cultured for 48 h with the indicated CHO transfectants (0.1×10^6/ml) with or without SEE (100 ng/ml). (B) Jurkat T cells were transiently transfected with an IL-2 enhancer-promoter (−500 to +60) driven by a luciferase reporter plasmid (pIL-2Luc). The Jurkat cells (10^6/ml) were further stimulated with the indicated CHO transfectants (0.1×10^6/ml) with or without SEE (100 ng/ml). Luciferase activity is expressed in arbitrary light units minus background units of buffer alone. Both sets of results shown are mean values from three experiments. Error bars indicate standard errors of the means. ▧, CHO; ▩, CHO-DR; ▨, CHO-DR/LFA-3; □, CHO-DR/B7-1; ■, CHO-DR/B7-1/LFA-3.

Moderate AP-1 binding activity, low NF-κB and NF-AT binding activity, but no CD28RE binding activity was observed after CHO-DR–SEE stimulation (Fig. 2 and 3A).

A slight enhancement of NF-κB and AP-1 binding activity, but no effect on the CD28RE, was seen after LFA-3 costimulation (Fig. 2). In contrast, these nuclear factors were strongly upregulated by B7-1 (Fig. 2). A further increase in the induction of the CD28RC was observed after B7-1–LFA-3 costimulation (Fig. 2A).

The nuclear levels of NF-AT were markedly upregulated by

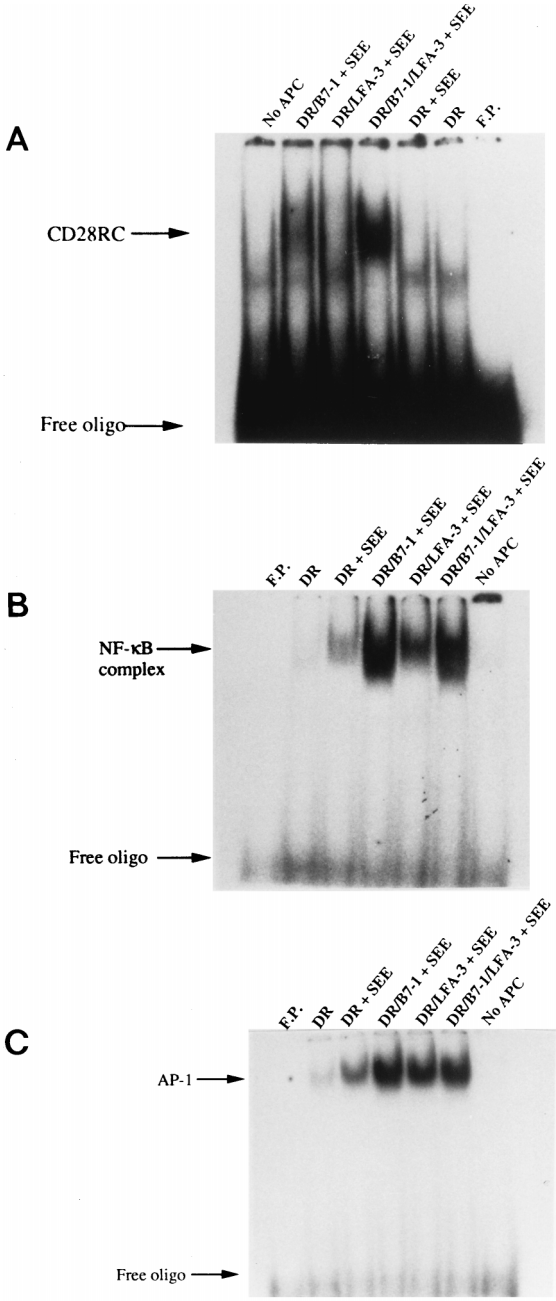

FIG. 2. Detectable levels of CD28RC (A), NF-κB (B), and AP-1 (C) complexes were observed in Jurkat T cells stimulated with SEE and CHO-DR/B7-1 or CHO-DR/B7-1/LFA-3. EMSA were performed with nuclear extracts from Jurkat T cells stimulated with CHO-DR, CHO-DR/B7-1, CHO-DR/LFA-3, and CHO-DR/B7-1/LFA-3 with and without SEE (100 ng/ml). Oligonucleotides (oligo) specific for the CD28RE and the NF-κB binding motifs were labeled with [γ-^{32}P]ATP and used for gel shift analysis. Results from one of two similar experiments are shown.

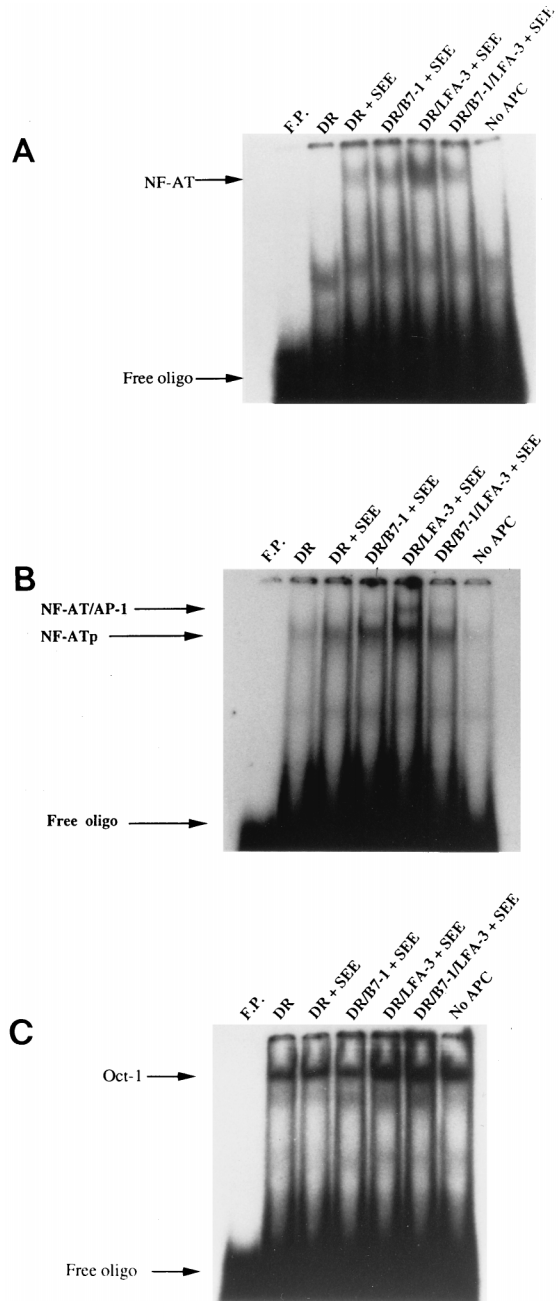

FIG. 3. Binding activities of hNF-AT (A), mNF-AT (B), and OCT-1 (C) in Jurkat T cells stimulated with SEE and CHO-DR/B7-1 or CHO-DR/B7-1/LFA-3. EMSA were performed with nuclear extracts from Jurkat T cells stimulated with CHO-DR, CHO-DR/B7-1, CHO-DR/LFA-3, and CHO-DR/B7-1/LFA-3 with or without SEE. Oligonucleotides (oligo) specific for the hNF-AT, mNF-AT, and AP-1 binding motifs were labeled with [γ-^{32}P]ATP and used for gel shift analysis. Results from one of two similar experiments are shown.

LFA-3 costimulation (Fig. 3A). In contrast, the increase in NF-AT binding activity induced by B7-1 was only marginal, indicating that NF-AT is a target nuclear factor for the CD2–LFA-3 activation pathway. NF-AT binding was only marginally increased after costimulation with CHO-DR/B7-1/LFA-3, suggesting that B7-1 costimulation may negatively regulate LFA-3-induced NF-AT DNA binding activity (Fig. 3A). CHO-DR–SEE stimulation increased slightly the levels of AP-1 and only marginally influenced NF-AT (Fig. 2C and 3A). A large amount of OCT-1 activity was observed in both unstimulated and stimulated Jurkat T cells, showing that all nuclear extracts were intact and contained similar amounts of nuclear proteins (Fig. 3C).

The mNF-AT sequence has been reported to give two gel shift bands upon activation, a lower band upon activation of NF-AT alone (NF-ATp) and an upper band upon activation of

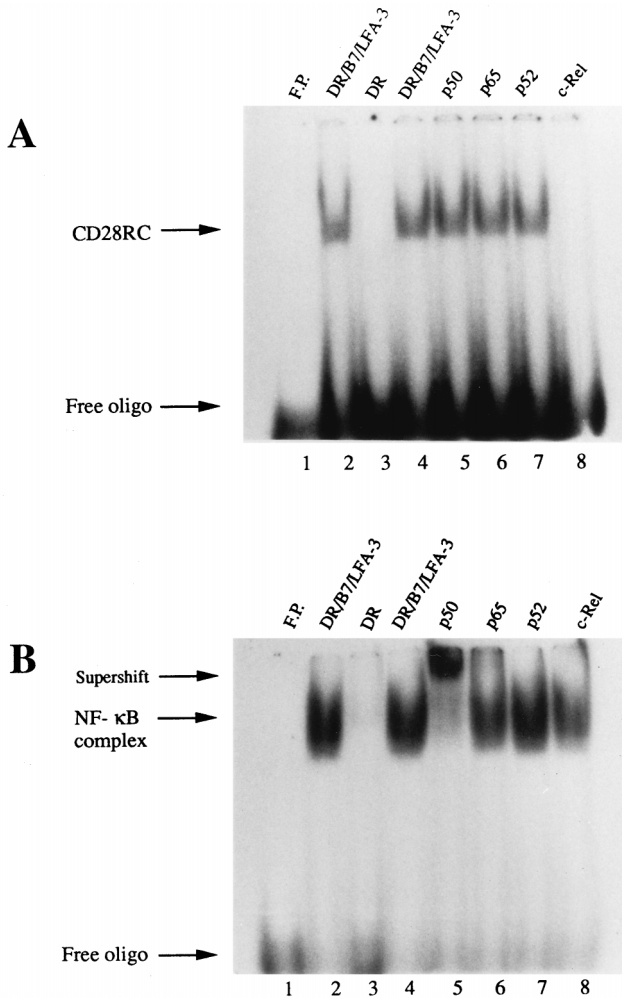

FIG. 4. Supershift analysis of CD28RC (A) and NF-κB (B) levels in Jurkat T cells stimulated with SEE and either CHO-DR/B7-1 or CHO-DR/B7-1/LFA-3. Nuclear protein extracts of Jurkat T cells stimulated with SEE (100 ng/ml) and either HLA-DR or HLA-DR–B7-1 were incubated with the different supershift Abs against the p50, p65, p52, and c-Rel proteins for 20 min on ice before addition of a ^{32}P-labeled CD28RC (A) and NF-κB (B). Lanes 1, free probe (F.P.); lanes 2, HLA-DR–B7-1 plus IgG control; lanes 3, HLA-DR without SEE; lanes 4, HLA-DR–B7-1-bound probe; lanes 5, HLA-DR–B7-1 plus p50; lanes 6, HLA-DR–B7-1 plus p65; lanes 7, HLA-DR–B7-1 plus p52; lanes 8, HLA-DR–B7-1 plus c-Rel. Results from one of two similar experiments are shown.

the NF-AT–AP-1 complex (29). Using the mNF-AT sequence, we found two complexes in Jurkat cells activated with CHO-DR/LFA-3, indicating the presence of NF-ATp and NF-AT–AP-1 complexes (Fig. 3B). Costimulation with the triple transfectant, CHO-DR/B7-1/LFA-3, negatively regulated both these components as shown by EMSA results (Fig. 3B). This supports the view that the observed negative regulation induced by B7-1–LFA-3 costimulation may involve downregulating NF-ATp as well as possibly influencing the composition of the AP-1 components.

To analyze the protein compositions of the different complexes induced in B7-1- and LFA-3-costimulated T cells, nuclear extracts from Jurkat T cells were treated with Abs that specifically recognize Rel family members or Fos and Jun proteins and analyzed by EMSA with NF-κB, CD28RC, hNF-AT, or AP-1 oligonucleotide. The studies revealed that the NF-κB complex induced by both HLA-DR–B7-1 and HLA-DR–LFA-3 was strongly supershifted by anti-p50 Ab (Fig. 4B, lane 5), partially blocked by anti-c-Rel Ab (Fig. 4B, lane 8), but only weakly influenced by Abs to p65 and p52 (Fig. 4B, lanes 6 and 7). This indicates that a major part of the complex in both LFA-3- and B7-1-costimulated T cells was p50-p50 homodimer. In contrast, the binding activity of the CD28RC in B7-1- and B7-1–LFA-3-costimulated T cells was completely abolished by the anti-c-Rel Abs (Fig. 4A, lane 8) but not recognized by p50, p65, or p52 Ab (Fig. 4A, lanes 5 to 7). These results demonstrate that the CD28RE, but not the NF-κB

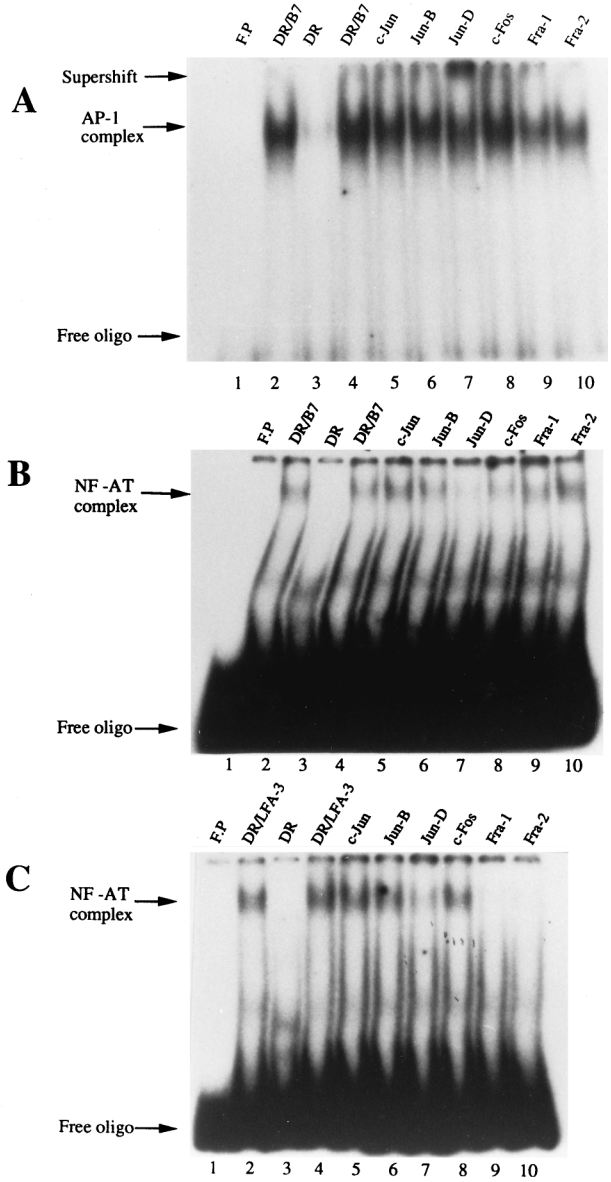

FIG. 5. Gel supershift assay for AP-1 and NF-AT complexes induced in B7-1- and LFA-3-costimulated Jurkat T cells. The AP-1 complex induced by B7-1 costimulation is shown (A). LFA-3 costimulation caused an identical composition. Distinct compositions of NT-AT complexes induced by B7-1 (B) and LFA-3 (C) are shown. Nuclear protein extracts of Jurkat T cells stimulated with SEE (100 ng/ml) and HLA-DR, HLA-DR–B7-1, and HLA-DR–LFA-3 were incubated with the different supershift Abs against the c-Jun, JunB, JunD, c-Fos, Fra-1, and Fra-2 proteins for 20 min on ice before addition of ^{32}P-labeled AP-1 (A) and NF-AT (B and C). Results from one of two similar experiments are shown.

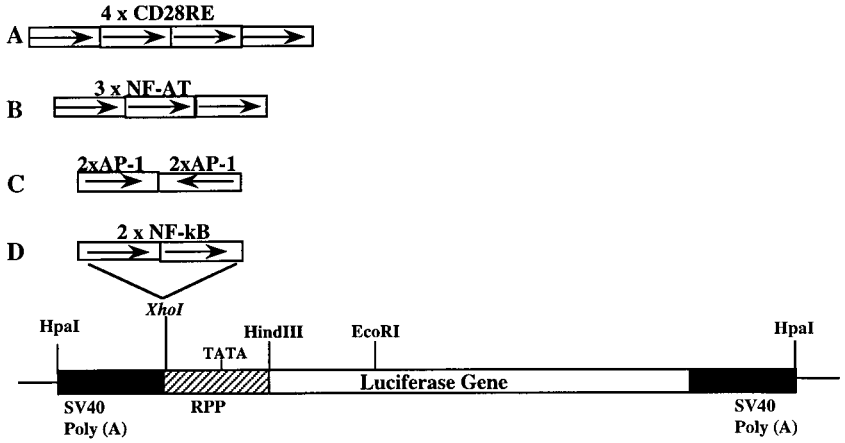

FIG. 6. Schematic representation of the luciferase reporter constructs containing tandem repeats of the CD28 (A), NF-AT (B), AP-1 (C), and NF-κB (D) binding sites. The sequences of the multimers were as described in the text.

response element, targets the binding of c-Rel nuclear factors in Jurkat cells (Fig. 4).

AP-1 supershift analysis showed the presence of substantial amounts of JunD proteins (Fig. 5A, lane 7). Fra-1- and Fra-2-reactive Abs partially blocked protein binding to the AP-1 response element (Fig. 5A, lanes 9 and 10), and minor blocking by c-Fos- and c-Jun-reactive Abs was also seen (Fig. 5A, lanes 5, 6, and 8). Identical compositions were seen in B7-1-, LFA-3-, and B7-1–LFA-3-costimulated T cells. In contrast, analysis of the AP-1 component of the NF-AT complex demonstrated that the compositions of this nuclear factor were distinct in B7-1- and LFA-3-costimulated cells. The binding of NF-AT complexes induced in Jurkat cells stimulated with HLA-DR–B7-1 and SEE was completely blocked by anti-JunD Abs (Fig. 5B, lane 7) and partially blocked by JunB and c-Fos Abs (Fig. 5B, lanes 6 and 8), but the NF-AT complexes were not recognized by c-Jun and Fra-1 Abs (Fig. 5B, lanes 5 and 10). Analysis of the nuclear proteins induced by HLA-DR–LFA-3 showed that NF-AT protein binding was completely abolished by anti-Fra-1 and anti-Fra-2 Abs (Fig. 5C, lanes 9 and 10), partially blocked by anti-JunD Abs (Fig. 5C, lane 7), and weakly influenced by anti-c-Fos Abs, but NF-AT protein was not recognized by the anti-c-Jun and anti-JunB Abs (Fig. 5C, lanes 5 and 6). These results demonstrate that B7-1- and LFA-3-costimulated T cells contain distinct AP-1 components binding to the NF-AT site in the IL-2 enhancer-promoter.

Functional analysis of NF-κB, CD28RE, AP-1, and NF-AT binding proteins. To determine the transcriptional activities of the NF-κB-, CD28RE-, AP-1-, and NF-AT-induced nuclear factors in LFA-3- and B7-1-costimulated cells, functional studies using luciferase as a reporter gene were performed with reporter gene constructs containing tandem repeats of CD28RE and the AP-1, NF-AT, and NF-κB response elements fused to the RPP (−36 to +37).

A schematic representation of the multimer constructs used in this study is displayed in Fig. 6. They contain the same oligonucleotide sequences as those used in the EMSA (see Materials and Methods).

Transient transfection of Jurkat cells revealed that the CD28RE (Fig. 7B) and the NF-κB (Fig. 7A) and AP-1 (Fig. 8B) response elements of the IL-2 enhancer were induced strongly in B7-1-costimulated T cells but only weakly in LFA-3-costimulated cells. NF-AT was strongly induced by both LFA-3 and B7-1 (Fig. 8A).

NF-κB- and NF-AT-driven transcription in costimulated Jurkat cells peaked at 4 h (Fig. 7A and 8A), while CD28RE- and AP-1-driven transcription peaked at 8 h (Fig. 7B and 8B). Notably, LFA-3 costimulation induced a transient NF-κB activity while B7-1-costimulated cells showed a strong and sustained response (Fig. 7A). Maximal AP-1-, NF-AT-, and CD28RE-driven transcription was seen in Jurkat cells costimulated with both LFA-3 and B7-1 (Fig. 7B and 8A and B). In contrast, NF-κB-dependent transcription was induced at optimal levels by B7-1 alone and not further augmented in B7-1–LFA-3-costimulated Jurkat T cells (Fig. 7A).

The CD28RE is required for B7-1- but not LFA-3-induced IL-2 transcription. Kinetic studies demonstrated that the IL-2 promoter-luciferase reporter gene activity peaked at 8 h after B7-1 costimulation. The IL-2 promoter luciferase response induced by LFA-3 was only marginal, while B7-1–LFA-3 superinduced the luciferase activity (Fig. 9A), suggesting a synergic effect of B7-1 and LFA-3 at the transcriptional level.

LFA-3, B7-1, and B7-1–LFA-3 costimulated IL-2 promoter-, AP-1-, and CD28RE-driven transcription in similar ways, which indicates a correlation between AP-1 and CD28RE levels and IL-2 superinduction in stimulated cells. Thus, LFA-3 induced a low, B7-1 a strong, and LFA-3–B7-1 a maximal response in T cells transiently transfected with these reporter gene constructs. The relative luciferase activity of the AP-1- and CD28RE-driven constructs was low compared to that of the NF-κB- and NF-AT-driven constructs. However, in the intact IL-2 promoter the CD28RE site is flanked by AP-1 sites, suggesting that CD28RE and AP-1 factors may act cooperatively to amplify IL-2 gene activity in vivo. To verify that the CD28RE plays a significant role in B7-1-induced IL-2 promoter transcription, we introduced a 6-bp mutation in the CD28RE sequence of a 500-bp IL-2 promoter. The mutation of the CD28RE site reduced the induction of IL-2 transcription by 80% in CHO-DR4/B7-1- and CHO-DR4/B7-1/LFA-3-costimulated T cells (Fig. 9B), while the low costimulatory activity induced by CHO-DR4/LFA-3 remained unaffected (Fig. 9B).

DISCUSSION

We recently reported that activation of human resting $CD4^+$ T cells with SEA and LFA-3 or B7-1 presented on CHO transfectants induced distinct cell activation profiles (43, 44). In this work we focused on the ability of B7-1 and LFA-3 costimulation to induce nuclear factors implicated in the reg-

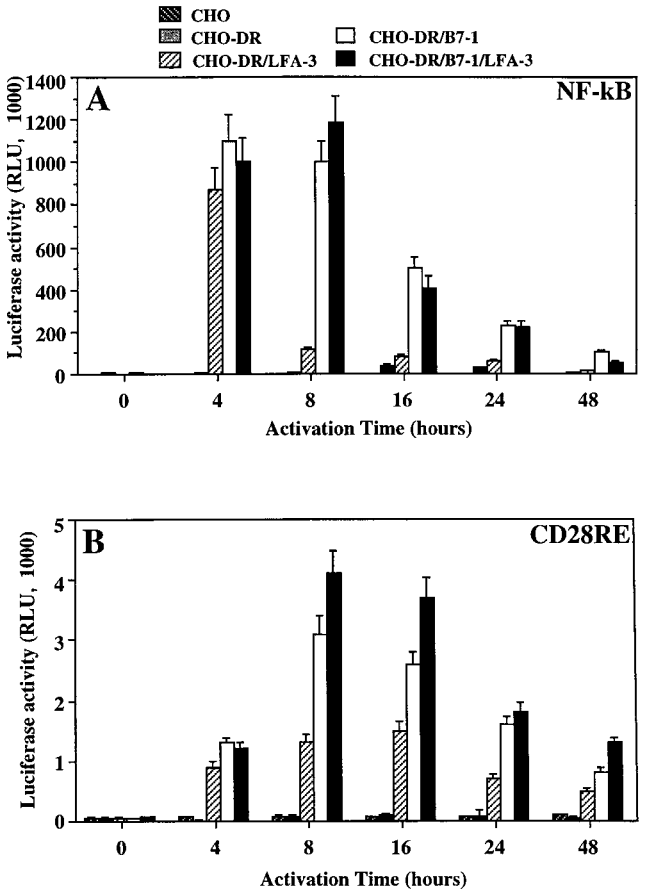

FIG. 7. Kinetics of NF-κB- and CD28RE-driven transcription. Reporter gene constructs containing tandem repeats of NF-κB and the CD28RE fused to the RPP (−36 to +37) and having the luciferase gene as the reporter gene were used. Jurkat T cells were transiently transfected with pNF-κB-Luc (two copies of NF-κB [GGGACTTTCC]) and pCD28RE-Luc (four copies of CD28RE [AAA GAAATTCC]) and then cultured in the presence of SEE and the different CHO-DR transfectants. Luciferase activity is expressed as arbitrary light units minus background units of buffer alone. Both sets of results shown are mean values from three similar experiments. Error bars indicate standard errors of the means.

ulation of the IL-2 gene promoter. Earlier studies have shown that CD28 costimulation may increase IL-2 production either by affecting IL-2 gene transcription or by prolonging IL-2 mRNA half-life (17, 38). Recently, Umlauf et al. (56) reported that B7 failed to increase IL-2 transcription but enhanced IL-2 mRNA stability in murine T cells, suggesting that mRNA stabilization is the major mechanism for B7-mediated IL-2 superinduction. We now show that in human Jurkat T cells, B7-1 costimulation induced a strong increase in IL-2 transcriptional activity. This discrepancy may reflect differences in murine and human T cells and the use of superantigen SEE versus CD3 monoclonal Ab to induce the first signal. A 40-fold increase in luciferase activity was observed after B7-1–SEE costimulation, and an 80-fold increase was seen in B7-1–LFA-3–SEE-costimulated cells. In contrast, the increase in activity induced by LFA-3 was only fivefold. This is compatible with our recent study on resting human peripheral blood $CD4^+$ T cells (43). LFA-3 was characterized as a weak inducer of IL-2 supporting autocrine T-cell growth, and B7-1 was characterized as a strong IL-2 inducer favoring paracrine cell growth (43). Although CD2 ligation induced small amounts of IL-2, this activity is sufficient to sustain autocrine proliferation and production of interferon and tumor necrosis factor (43). We now show that maximal induction of the NF-AT complex required LFA-3 costimulation, indicating that the NF-AT complex may be a target factor for the autocrine CD2–LFA-3 signal pathway. The NF-AT complex contains both AP-1 and NF-ATp (10, 26, 27). Most interestingly, the AP-1–NF-AT components in LFA-3- and B7-1-costimulated cells differed qualitatively. The NF-AT protein has been regarded as a Ca^{2+}-dependent nuclear factor induced by engagement of the TCR. This is the first report to our knowledge that has demonstrated a specific effect of the CD2–LFA-3 pathway in the modulation of NF-AT activity, suggesting that the TCR-CD3 and CD2 pathways may cross-talk at this level. NF-AT-dependent transcriptional activity requires both a CsA-sensitive translocation of NF-AT from the cytosol to the nucleus and a CsA-insensitive stimulation-dependent step which involves de novo synthesis of the Fos protein (7, 40) and posttranslational modification of Jun proteins. The use of an mNF-AT element, which forms two distinct bands in gel shift assays (29) since it binds NF-ATp on its own as well as in complex with AP-1, caused an increased nuclear level of NF-ATp in LFA-3-costimulated T cells. This suggests that LFA-3 costimulation enhances the amount of

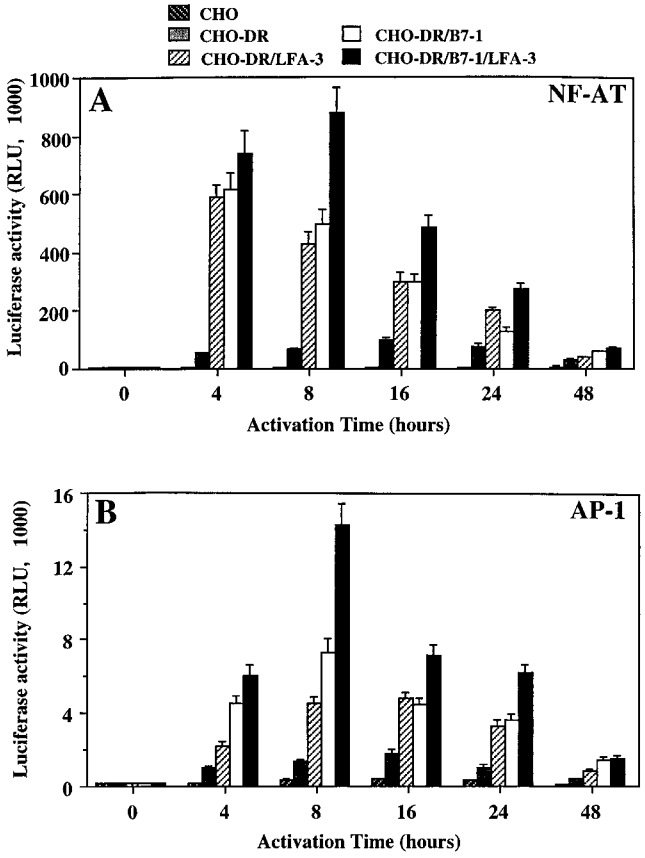

FIG. 8. Kinetics of induction of NF-AT and AP-1 transcription factors. Transcription was analyzed with reporter gene constructs containing tandem repeats of NF-AT and AP-1 fused to the RPP (−36 to +37) and having the luciferase gene as the reporter gene. Jurkat T cells were transiently transfected with pNF-AT-Luc (three copies of NF-AT [GGAGAAAAACTGTTTCATACAGA AGGC]) and pAP-1-Luc (four copies of AP-1 [CGATTGAGTCAGGGTAAC GG]) and then cultured in the presence of SEE and the different CHO-DR transfectants. Luciferase activity is expressed as arbitrary light units minus background units of buffer alone. Both sets of results shown are mean values from three similar experiments. Error bars indicate standard errors of the means.

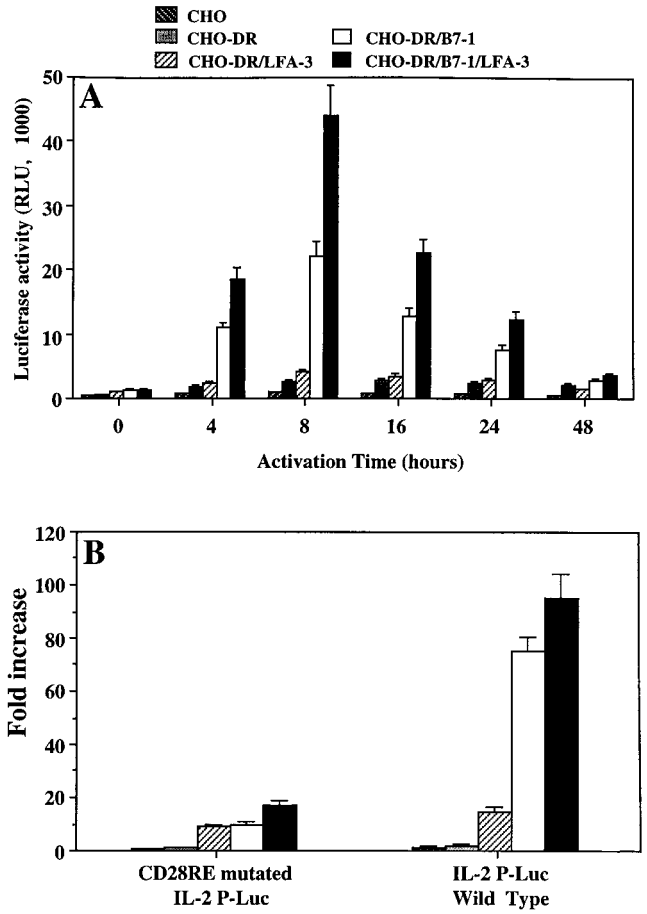

FIG. 9. Transcription of the IL-2 enhancer-promoter region. (A) Kinetics of the native IL-2 promoter. Jurkat T cells were transiently transfected with the IL-2 reporter gene, and the cells were harvested for luciferase assay at various time points after costimulation with SEE and the different CHO transfectants. (B) Activity of a mutated CD28RE IL-2 enhancer. Jurkat T cells were transfected with the wild-type IL-2 promoter or the CD28RE mutant promoter, and the cells were stimulated with SEE and the different CHO transfectants. Eight hours later, samples were harvested and analyzed for luciferase activity. The native CD28RE sequence of the IL-2 promoter (−164 to −154, AAAGAAAT TCC) was mutated to the AAAG**ACCTCGA** sequence (mutation shown in boldface). Luciferase activity is expressed as arbitrary light units minus background units of buffer alone (A) or as fold activation over the value obtained for unstimulated cells (B). Both sets of results shown are mean values from four similar experiments. Error bars indicate standard errors of the means.

available nuclear NF-ATp and may involve modulation of NF-AT translocation from the cytosol to the nucleus. LFA-3 induced higher levels of NF-AT binding proteins in gel shift assays than did B7-1–LFA-3, suggesting that expression of B7-1 negatively regulates NF-AT binding activity. This possibility may be due to the observed different AP-1 subcomponents of the NF-AT complexes in B7-1- and LFA-3-stimulated cells. Alternatively, B7-1 may interfere with LFA-3-induced mobilization of NF-ATp proteins. Indeed, using mNF-AT response element signal transduction, we found that LFA-3 costimulation increased both the NF-ATp and NF-AT–AP-1 complexes to a greater extent than B7-1–LFA-3 stimulation, implying that combined B7-1 and LFA-3 costimulation negatively regulated these two complexes. Analyses of NF-AT-driven transcription demonstrated a similar effect of B7-1 and LFA-3 on NF-AT-dependent transcription. This is in contrast to the gel shift studies showing strong NF-AT binding activity after LFA-3 but not B7-1 costimulation. Since the AP-1 subcomponents of the NF-AT complexes differed in LFA-3- and B7-1-induced cells, the results suggest that the JunD, Fra-1, and Fra-2 complexes seen in B7-1-costimulated cells may have favorable effects on transcriptional activity. Similarly, combined B7-1 and LFA-3 costimulation augmented NF-AT-dependent transcription but did not increase the amount of NF-AT binding activity as analyzed by EMSA. This supports the notion that these pathways cross-talk to optimize the transcriptional activity of the NF-AT–AP-1 complex.

Earlier reports indicated a central role for the CD28RE in CD28-costimulated T cells, although recent reports suggested that the CD28RC was not exclusively seen in CD28-stimulated T cells (9, 19). Using native B7-1 and LFA-3 ligands, we have now found that the CD28RC is detectable in EMSA only after B7-1 costimulation. However, the presence of LFA-3 seems to contribute to the induction of maximal binding levels in CD28RC- and CD28RE-dependent transcription. Thus, the complete lack of specificity reported in other studies may reflect the use of nonphysiologic ligands, such as phorbol myristate acetate and phytohemagglutinin (9, 19). The CD28RC observed in gel shift analysis was completely blocked with c-Rel Abs but not with p50, p65, or p52 Abs, which suggests the presence of a c-Rel homodimer. Alternatively, c-Rel might be present as an essential component but bound to a non-Rel protein in the complex. Indeed, recent studies have implied that the NF-AT protein, which is structurally related to the Rel family, may bind to CD28RE-containing oligonucleotides (28). Additional EMSA studies using the mutated CD28RE sequence showed a loss of CD28RE binding activity in extracts from B7-1- or B7-1–LFA-3-costimulated Jurkat T cells (data not shown). This confirms earlier studies (17, 18) demonstrating that the core sequence GAAATTCC is required for both CD28RC binding and transcriptional activity. The dramatic reduction in transcriptional IL-2 activity observed with an IL-2 promoter-reporter construct containing a CD28RE mutant site further underlined the functional importance of the CD28RE. Apparently, induction of c-Rel containing the CD28RC in CD28-ligated T cells is required to provide an optimal second signal. The results also demonstrate that the mutation in the CD28RE severely affects the cross-talking between the CD2 and CD28 pathways.

Interestingly, LFA-3 costimulation was relatively unaffected by the CD28RE mutation, suggesting that LFA-3-driven IL-2 transcription is independent of CD28RE binding activity. The CD28RE is flanked by AP-1 sites in the IL-2 promoter (18). The observation that the levels of AP-1 and the CD28RC in B7-1-, LFA-3-, and B7-1–LFA-3-costimulated cells correlate with IL-2 transcription makes it tempting to speculate that the c-Rel and AP-1 proteins interact to form an integrated transcriptionally active unit in the IL-2 promoter in vivo. Although LFA-3 to a certain degree induced transcription of the reporter gene having four CD28REs, the low level of IL-2 transcription and the resistance to the CD28RE mutation observed in LFA-3-costimulated cells support the notion that induction of this AP-1–CD28RE complex in appropriate amounts and with the appropriate composition is seen only in CD28-stimulated T cells. For excessive induction of IL-2 gene activity, the binding of the Rel complexes induced via CD28 alone or by combined CD28–CD2 stimulation most likely is not sufficient since the relative activity of the reporter with four CD28REs is comparatively modest. This implies that activation of other regulatory elements may be needed for CD28-mediated IL-2 superinduction. The nuclear factor AP-1 may be a candidate for B7-1-mediated costimulation. It is possible that certain AP-1 components can stabilize CD28RC binding and facilitate

transcription. Indeed, there are precedents for AP-1 cooperatively binding with several nuclear factors (10, 13), including NF-AT and Rel proteins (53). The suggested AP-1–c-Rel cooperativity may involve modulation of protein-protein interactions similar to that by NF-AT and AP-1 or a functional regulation of transcription by AP-1 and c-Rel acting in a *cis* configuration. The ability of Jun and Fos (AP-1) proteins to cross-couple with NF-κB in other studies (53) supports the hypothesis of cross-talk between these nuclear factors on the IL-2 promoter in B7-1-costimulated T cells. Further studies are required to characterize the components binding to the AP-1–CD28–AP-1 sequence and their molecular interactions.

The present study demonstrates unique effects of B7-1 and LFA-3 on the induction of AP-1, CD28RC, NF-κB, and NF-AT. The strong induction of NF-AT but the induction of moderate levels of AP-1 and transient levels of NF-κB in LFA-3-costimulated T cells may contribute to limit the level of IL-2 gene transcription and favor autocrine proliferation. The transition to a paracrine T-cell response (43) during B7-1 costimulation most likely involves enhanced levels of AP-1 and the CD28RC and a sustained expression of NF-κB. The coexpression of B7-1 and LFA-3 on activated professional antigen-presenting cells contributes to induction of large amounts of AP-1 and the CD28RC, increased transcriptional activity of the AP-1–NF-AT complex, and superinduction of IL-2 production. Thus, mobilization of signal transduction pathways augmenting both Rel proteins and Fos and Jun proteins seems to be critical for IL-2 superinduction.

ACKNOWLEDGMENTS

We gratefully acknowledge C. Verweij (Leiden, The Netherlands) for providing Jurkat T cells, D. Sansom (Bath, United Kingdom) for providing CHO transfectants, and M. Rincón and R. A. Flavell (Section of Immunology, Howard Hughes Medical Institute, Yale University School of Medicine) for the AP-1–luciferase reporter plasmid.

This work was supported by grants from the Swedish Cancer Society and the LEO Research Foundation.

REFERENCES

1. **Allison, J. P.** 1994. CD28/B7 interaction in T cell activation. Curr. Opin. Immunol. **6:**414–419.
2. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49:**729–739.
3. **Beg, A. A., and A. S. Baldwin.** 1993. The IκB proteins: multifunctional regulators of Rel/NF-κB transcription factors. Genes Dev. **7:**1564–1570.
4. **Bierer, B. E., S. Herrmann, and S. J. Burakoff.** 1988. Interaction of CD2 with its ligand, LFA-3 in human T cell proliferation. J. Immunol. **140:**3358–3362.
5. **Bours, V., G. Franzoso, V. Azarenko, S. Park, T. Kanno, K. Brown, and U. Siebenlist.** 1993. The oncoprotein Bcl-3 directly transactivates through κB motifs via association with DNA-binding p50B homodimers. Cell **72:**729–739.
6. **Boussiotis, V. A., G. J. Freeman, J. D. Griffin, G. S. Gray, J. G. Gribben, and L. M. Nadler.** 1994. CD2 is involved in maintenance and reversal of human alloantigen-specific clonal anergy. J. Exp. Med. **180:**1665–1673.
7. **Brabletz, T., I. Pietrowsky, and E. Serfling.** 1991. The immunosuppressive FK-506 and cyclosporin A inhibit the generation of protein factors binding to the two purine boxes of the interleukin 2 enhancer. Nucleic Acids Res. **19:**61–67.
8. **Brown, K., S. Gerstberger, L. Carlson, G. Franzoso, and U. Siebenlist.** 1995. Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science **267:**1485–1488.
9. **Bryan, R. G., Y. Li, J.-H. Lai, M. Van, N. R. Rice, R. R. Rich, and T.-H. Tan.** 1994. Effect of CD28 signal transduction on c-Rel in human peripheral blood T cells. Mol. Cell. Biol. **14:**7933–7942.
10. **Castigli, E., T. A. Chatila, and R. S. Geha.** 1993. A protein of the AP-1 family is a component of nuclear factor of activated T cells. J. Immunol. **150:**3284–3290.
11. **Choi, M. S. K., R. D. Brines, M. J. Holman, and G. G. B. Klaus.** 1994. Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. Immunity **1:**179–187.
12. **Civil, A., M. Geerts, L. A. Aarden, and C. L. Verweij.** 1992. Evidence for a role of CD28RE as a response element for distinct mitogenic T cell activation signal. Eur. J. Immunol. **22:**3041–3043.
13. **De Grazia, U., M. P. Felli, A. Vacca, A. R. Farina, M. Maroder, L. Cappabianca, D. Meco, M. Farina, I. Screpanti, L. Frati, and A. Gulino.** 1994. Positive and negative regulation of the composite octamer motif of the interleukin 2 enhancer by AP-1, Oct-2, and retinoic acid receptor. J. Exp. Med. **180:**1485–1497.
14. **Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8:**1715–1724.
15. **Dustin, M., M. Sanders, S. Shaw, and T. Springer.** 1987. Purified lymphocyte function-associated antigen 333 binds to CD2 and mediates T lymphocyte adhesion. J. Exp. Med. **165:**677–692.
16. **Fischer, H., G. Hedlund, T. Kalland, H. O. Sjögren, and M. Dohlsten.** 1990. Independent regulation of IFN-γ and tumor necrosis factor by IL-1 in human T helper cells. J. Immunol. **145:**3767–3771.
17. **Fraser, J. D., and A. Weiss.** 1992. Regulation of T-cell lymphokine gene transcription by the accessory molecule CD28. Mol. Cell. Biol. **12:**4357–4363.
18. **Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss.** 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. Science **251:**313–316.
19. **Ghos, P., T. Tan, N. R. Rice, A. Sica, and H. A. Young.** 1993. The interleukin 2 CD28-responsive complex contains at least three members of the κB family: c-Rel, p50 and p65. Proc. Natl. Acad. Sci. USA **90:**1696–1700.
20. **Gjörloff, A., G. Hedlund, T. Kalland, D. Sansom, H. Fischer, J. Trowsdale, H. O. Sjögren, and M. Dohlsten.** 1992. The LFA-3 adhesion pathway is differently utilized by superantigen-activated human $CD4^+$ T cell subsets. Scand. J. Immunol. **36:**243–250.
21. **Granelli-Piperno, A., and P. Nolan.** 1991. Nuclear transcription factors that bind to elements of the IL-2 promoter. J. Immunol. **147:**2734–2739.
22. **Grilli, M., J.-S. Jason, and M. J. Lenardo.** 1993. NF-κB and rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol. **143:**1–62.
23. **Grimm, S., and P. A. Baeuerle.** 1993. The inducible transcription factor NF-κB: structure-function relationship of its protein subunits. Biochem. J. **290:**297–308.
24. **Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raluet, and J. P. Allison.** 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. Nature **356:**607–609.
25. **Hay, R. T.** 1993. Control of nuclear factor-κB DNA binding activity by inhibitor proteins containing ankyrin repeats. Biochem. Soc. Trans. **21:**926–930.
26. **Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao.** 1992. Nuclear factor of activated T cells contains Fos and Jun. Nature **356:**801–804.
27. **Jain, J., P. G. McCaffrey, Z. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao.** 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature **365:**352–355.
28. **Jain, J., E. Burgeon, T. M. Badalian, P. G. Hogan, and A. Rao.** 1995. A similar DNA-binding motif in NF-AT family proteins and the Rel homology region. J. Biol. Chem. **270:**4138–4145.
29. **Jain, J., Z. Miner, and A. Rao.** 1993. Analysis of the preexisting and nuclear forms of nuclear factor of activated T cells. J. Immunol. **151:**837–848.
30. **Janeway, C.** 1989. Immunogenicity signals 1, 2, 3 . . . and 0. Immunol. Today **10:**283–286.
31. **Janeway, C. A., and M. Golstein.** 1993. Lymphocyte activation and effector functions. The role of cell surface molecules. Curr. Opin. Immunol. **5:**313–323.
32. **Jenkins, M. K., and J. G. Johnson.** 1993. Molecules involved in T-cell costimulation. Curr. Opin. Immunol. **5:**361–367.
33. **June, C. H., A. Jeffrey, A. Ledbetter, P. S. Linsley, and C. B. Thompson.** 1990. Role of the CD28 receptor in T-cell activation. Immunol. Today **11:**211–216.
34. **Kamps, M. P., L. Corcoran, J. H. LeBowitz, and D. Baltimore.** 1990. The promoter of the human interleukin-2 gene contains two octamer-binding sites and is partially activated by the expression of Oct-2. Mol. Cell. Biol. **10:**5464–5472.
35. **Koyasu, S., T. Lawton, D. Novick, M. A. Recny, F. Siliciano, B. P. Wallner, and E. L. Reinherz.** 1990. Role of interaction of CD2 molecules with lymphocyte function-associated antigen 3 in T-cell recognition of nominal antigen. Proc. Natl. Acad. Sci. USA **87:**2603–2607.
36. **Lai, J.-H., G. Horvath, J. Subleski, J. Bruder, P. Ghosh, and T.-H. Tan.** 1995. RelA is a potent transcriptional activator of the CD28 response element within the interleukin 2 promoter. Mol. Cell. Biol. **15:**4260–4271.
37. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcriptional factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49:**741–752.
38. **Lindsten, T., C. June, J. A. Ledbetter, G. Stella, and C. B. Thompson.** 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. Science **244:**339–343.
39. **Liou, H. C., and D. Baltimore.** 1993. Regulation of the NF-κB/rel transcription factor and IκB inhibitor system. Curr. Opin. Cell Biol. **5:**477–487.
40. **Mattila, P. S., K. S. Ullman, S. Fiering, E. A. Emmel, M. McCutcheon, G. R. Crabtree, and L. A. Herzenberg.** 1990. The actions of cyclosporin A and

FK-506 suggest a novel step in the activation of T lymphocytes. EMBO J. **9:**4425–4433.
41. **McCaffrey, P. G., C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran, G. L. Verdine, A. Rao, and P. G. Hogan.** 1993. Isolation of the cyclosporin-sensitive T cell transcription factor NF-ATp. Science **262:**750–754.
42. **Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, L. A. Timmerman, G. P. Nolan, A. Admon, and G. R. Crabtree.** 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature **369:**497–502.
43. **Parra, E., A. Gjörloff-Wingren, G. Hedlund, M. Björklund, H. O. Sjögren, T. Kalland, D. Sansom, and M. Dohlsten.** 1994. Costimulation of human CD4+ T lymphocytes with B7 and LFA-3 results in distinct cell activation profiles. J. Immunol. **153:**2479–2487.
44. **Parra, E., M. Varga, M. Sigvardsson, G. Hedlund, T. Kalland, T. Leanderson, H. O. Sjögren, and M. Dohlsten.** 1995. Costimulation of human $CD4^+$ T cells with LFA-3 and B7 induce distinct effects on AP-1 and NF-κB transcription factors. J. Immunol. **155:**1132–1140.
45. **Rao, A.** 1994. NF-ATp: a transcription factor required for the coordinate induction of several cytokine genes. Immunol. Today **15:**274–281.
46. **Rincón, M., and R. A. Flavell.** 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. EMBO J. **13:**4370–4381.
47. **Rooney, J. W., Y.-L. Sun, L. H. Glimcher, and T. Hoey.** 1995. Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. Mol. Cell. Biol. **15:**6299–6310.
48. **Samelson, L. E.** 1989. Lymphocyte activation. Curr. Opin. Immunol. **2:**210–214.
49. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with "mini-extracts", prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
50. **Schwartz, R. H.** 1992. Costimulation of T lymphocytes: the role of CD28, CTLA-4 and B7/BB1 in interleukin-2 production and immunotherapy. Cell **71:**1065–1068.
51. **Sen, R., and D. Baltimore.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705–716.
52. **Shaw, J.-P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree.** 1988. Identification of a putative regulator of early T cell activation genes. Science **241:**202–205.
53. **Stein, B., A. S. Baldwin, Jr., D. W. Ballard, W. C. Greene, P. Angel, and P. Herrlich.** 1993. Cross-coupling of the NF-κB p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J. **12:**3879–3891.
54. **Traeckner, E. B.-M., H. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Baeuerle.** 1995. Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J. **14:**2876–2883.
55. **Turcovski-Corrales, S. M., R. G. Fenton, G. Peltz, and D. D. Taub.** 1995. CD28:B7 interaction promote T cell adhesion. Eur. J. Immunol. **25:**3087–3093.
56. **Umlauf, S. W., B. Beverly, S.-M. Kang, K. Brorson, A.-C. Tran, and R. H. Schwartz.** 1993. Molecular regulation of the IL-2 gene: rheostatic control of the immune system. Immunol. Rev. **133:**177–195.
57. **Venkataraman, L., D. A. Francis, Z. Wang, J. Liu, T. L. Rothstein, and R. Sen.** 1994. Cyclosporin-A sensitive induction of NF-AT in murine B cells. Immunity **1:**189–196.
58. **Verweij, C. L., M. Geerts, and L. A. Aarden.** 1991. Activation of interleukin-2 gene transcription via the T-cell surface molecule CD28 is mediated through an NF-κB-like response element. J. Biol. Chem. **266:**14179–14182.
59. **Yiling, L., A. Granelli-Piperno, J. M. Bjorndahl, C. A. Phillips, and J. M. Trevillyan.** 1992. CD28-induced T cell activation. J. Immunol. **149:**24–29.