Differential Induction of Transcription Factors That Regulate the Interleukin 2 Gene during Anergy Induction and Restimulation

By Cindy Go and Jim Miller

From the Departments of Pathology and Molecular Genetics and Cell Biology, University of Chicago, Chicago, Illinois 60637

Summary

T cell activation requires two distinct signals. The first is delivered through the antigen-specific T cell receptor (TCR), and the second is provided by costimulatory molecule(s) present on the surface of the antigen-presenting cell (APC). Stimulation of T helper type 1 T cell clones through the TCR in the absence of the costimulatory activity results in a lack of interleukin 2 (IL-2) secretion and proliferation, and the induction of a long-lived state of nonresponsiveness, termed anergy. In this study, we have examined the transcription factors involved in IL-2 gene expression that are expressed after stimulation of normal T cell clones through the TCR with and without engagement of the necessary costimulatory molecule(s). Antigen-specific activation of the clones results in the induction of a similar pattern of transcription factors that have been previously shown to regulate IL-2 expression. In contrast, antigen presentation by chemically fixed APC, a condition that results in T cell anergy, induces neither NF-AT nor one of the two NF-κB binding factors. Thus, the failure to express IL-2 during the induction of T cell anergy may be attributed to the absence of these two transcription factors. When anergized T cells are restimulated with antigen and conventional APC, they induce the transcription factors associated with IL-2 expression, but they fail to synthesize measurable IL-2. Taken together, these data indicate that the control of IL-2 gene expression during anergy induction and during normal stimulation of anergized cells are distinct, and suggest the presence of additional regulatory elements in the IL-2 gene.

Antigen-specific T cell activation requires corecognition of foreign antigen and self-MHC molecules on the surface of APCs. Soluble protein antigens are internalized by APC, intracellularly processed by denaturation and/or proteolysis, and presented at the cell surface in a stable complex with MHC class II molecules. It is this class II:peptide complex that is recognized by the TCR on $CD4^+$ T cells (1). In addition, T cell activation requires a second signal that is provided by the APC (2). Recently, several candidate molecules expressed by APC have been proposed to function as costimulatory molecules for T cell activation. The best documented case is for B7, the ligand for CD28 (3–7), but a role for intercellular adhesion molecule (ICAM)-1/LFA-1 (8), VCAM-1/VLA-4 (8), fibronectin/VLA-4/5 (9), and CD2/LFA-3 (10) have also been suggested.

The requirement for accessory signals is less well established for the Th2 subset of $CD4^+$ T cells (11), although the ability of these cells to provide their own accessory function in vitro may mask this requirement (12). In the case of Th1 cells, there appears to be a strict requirement for this accessory function. Stimulation of Th1 T cell clones through the TCR in the absence of costimulatory signals results in low levels of IFN-γ and IL-3 expression, a failure to transcribe and secrete IL-2, and a failure to proliferate. Furthermore, engagement of the T cell receptor in the absence of appropriate second signals can lead to clonal inactivation, and a long-lived state of anergy (2, 13–15). Experimentally, anergy can be induced by stimulation of T cell clones with chemically fixed, conventional APCs and antigen (13), purified class II MHC-peptide complexes inserted into planar membranes (15), nonconventional APCs, such as islet cells (16) or keratinocytes (17), or by stimulation with antibodies directed against the TCR/CD3 complex (11, 18). Once anergized, Th1 clones will remain viable and will proliferate to exogenous IL-2, but they will not synthesize IL-2 nor proliferate when stimulated with nominal antigen and competent APCs (2, 14).

In this report, we have examined the expression of some of the nuclear factors that regulate the transcription of the IL-2 gene during T cell anergy induction and during restimulation of anergized cells. We have focused our analysis on the IL-2 gene for two reasons. First, the expression of IL-2 is crit-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/05/1327/10 $2.00
Volume 175 May 1992 1327–1336

ical for the function of the Th1 subset of T cells. Second, the expression of IL-2 is strictly dependent on the presence of accessory molecules during T cell activation, and appears to be completely repressed in anergized cells. The IL-2 promoter/enhancer has been carefully described (19, 20, 21–26), and consists of several regulatory regions, one of which binds a T cell–specific transcription factor, NF-AT, another that binds a lymphoid specific factor (NF-κB), and other regions that bind more general transcription factors. Therefore, we have assayed the expression of these factors during stimulation of T cells in the presence and absence of accessory function. We find that several nuclear factors that have been shown to be critical in the regulation of the IL-2 gene are not induced by T cells in the absence of accessory molecules. In contrast, once cells are anergized, they can express all of the relevant transcription factors after stimulation in the presence of accessory function, but do not express any IL-2. These data suggest that the failure to express IL-2 in the absence of accessory molecules may be due to the generation of an incomplete transcription complex at the IL-2 promoter, and suggest that additional regulatory factors must be involved in repressing the IL-2 gene in anergized cells.

Materials and Methods

Cell Lines. Th1 clones, pGL2 and pGL10, were obtained from Frank Fitch (27), and are specific for OVA peptide 323–339 in the context of MHC class II, I-A^d. A tryptic digest of OVA was used as a source of this peptide (28). Clones were maintained by weekly passage with irradiated BALB/cJ splenocytes, 400 μg/ml OVA (Sigma Chemical Co., St. Louis, MO), 20 U/ml IL-2 (Amgen, Thousand Oaks, CA), and 200 U/ml IFN-γ (Amgen) in DMEM supplemented with 10% FCS (Hyclone Laboratories, Inc., Logan, UT), 10 mM Hepes, 2 mM glutamine, 0.1 mM nonessential amino acids (Gibco Laboratories, Grand Island, NY), and 50 μM 2-ME (Sigma Chemical Co.). Clones were rested a minimum of 8 d post-stimulation before use in assays.

Mice. BALB/cJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6–8-wk of age.

Induction of Anergy. BALB/cJ splenocytes were depleted of RBC by lysis in 17 mM Tris (pH 7.4), 144 mM NH_4Cl, and of T cells by staining with the anti-Thy 1 mAb, G7 (29), and treatment with Rabbit complement (Accurate Chemicals, Westbury, NY). These T-depleted splenocytes were fixed in a 1% paraformaldehyde solution in HBSS (Gibco Laboratories) for 20 min at room temperature, and washed three times with complete media, incubated 30 min at 37°C in complete media, and washed three additional times with complete media. Th1 clones were Ficoll purified and incubated overnight with a 10-fold excess of paraformaldehyde-fixed, T cell–depleted splenocytes plus 250 μg/ml tryptic OVA digest at a density of 2.5×10^5 T cells/cm^2 in 100-mm tissue culture dishes for 16 h at 37°C. T cells were activated under the same conditions, except that irradiated, T cell–depleted splenocytes (2,000 rad) were used as APC.

Nuclear Extracts. T cells were harvested from the tissue culture dishes and purified on a Ficoll-hypaque gradient to remove the APCs. To further purify the T cell clone, the remaining cells were stained with anti-IA^d mAb, MKD6 (30), and panned on goat anti–mouse IgG (Jackson Immunoresearch Labs, Inc., West Grove, PA) coated plates. An aliquot of purified T cells were assayed for anergy induction as described below. The remainder were used to isolate nuclear extracts for gel shift assays. Nuclear extracts were made according to the method described by Fiering et al., with the exception that the extracts were not passed over a P6DG column (31). Briefly, cells were allowed to swell in buffer A (10 mM Hepes, pH 7.8, 15 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 1 μg/ml Antipain, 0.3 μg/ml Leupeptin) for 1 min on ice and pelleted for 3 min at 3,000 rpm in a microfuge. Cells were then lysed in buffer A plus 0.2% NP-40. Nuclei were collected by centrifugation as above and resuspended in Buffer B (50 mM Hepes, pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10% glycerol). Ammonium sulfate was added to a final concentration of 0.3 M, and tubes were rocked in a cold room for 30 min. Nuclear debris and membranes were pelleted at 100,000 *g* for 10 min in an airfuge (Beckman Instruments Inc., Fullerton, CA). Ammonium sulfate was added to the supernatant to a final concentration of 1.69 M, and incubated on ice for 5 min. Nuclear proteins were pelleted at 50,000 *g* for 5 min in the airfuge. Precipitated proteins were resuspended in Buffer B and used for gel mobility shift assays. Protein concentration of extracts was measured by the BioRad protein assay kit (Bio-Rad Laboratories, Richmond, CA) and checked for integrity by silver stain of SDS/PAGE.

Gel Mobility Shift Assay. The oligonucleotides used were monomers of the binding sites: NFIL-2A (−93 to −63 from human IL-2), AP-1 consensus site (32), NF-κB consensus site (33), and NF-AT (−285 to −255 from human IL-2). The binding reactions were performed essentially as described (34, 35) with the same amount of protein in each binding reaction (2–4 μg) in a solution consisting of 10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, and 4 μg poly dI-dC (Pharmacia Fine Chemicals, Piscataway, NJ). The reactions were incubated at room temperature for 20 min with 10,000 cpm double-stranded ^{32}P-labeled oligonucleotides. The samples were electrophoresed on 4% polyacrylamide gels in a buffer consisting of 25 mM Tris, 190 mM glycine, and 1 mM EDTA.

Lymphokine Assay. IL-2 was assayed with the indicator line CTLL-4 on threefold dilutions of supernatant by the colorimetric MTT assay (36). IL-3 was assayed with the indicator cell line FDCP1-IR (37, 38) on threefold dilutions of supernatant by the colorimetric MTT assay. IFN-γ was assayed by ELISA (39).

Restimulation of Anergic T Cells. 2.5×10^4 pretreated T cells were incubated in 96-well plates at 37°C with 2.5×10^5 irradiated BALB/cJ splenocytes in varying doses of antigen. After 48 h, plates were pulsed with 1 μCi [^3H]thymidine/well and harvested the next day. Counts are represented as cpm × 10^3.

Results

Occupancy of the TCR in the Absence of Accessory Function Fails to Induce IL-2 Secretion and Proliferation in Th1 Clones. We have used two Th1 clones, pGL2 and pGL10, with the same peptide (OVA peptide 323–339) and MHC class II (I-A^d) specificity, but derived from two different strains of mice. These clones were incubated overnight with peptide antigen presented by either fixed or irradiated T cell–depleted splenocytes. As expected, stimulation of the T cells with conventional, irradiated APCs resulted in vigorous proliferation and lymphokine production of IL-2, -3, and IFN-γ, but not IL-4 ([27], Table 1). The two Th1 clones inherently produced different levels of the three lymphokines after activation, but the patterns observed were similar for each clone. In contrast to normal activation, stimulation of the clones with fixed APC and antigen resulted in suboptimal production of IL-3

Table 1. *Lymphokine Production and Proliferation of Th1 Clones after Antigen Presentation by Fixed and Irradiated APCs*

Cell line	Culture condition	Lymphokine				Proliferation
		IL-2	IL-3	IFN-γ	IL-4	
		U/ml				*cpm × 10³*
pGL2	Rest	0	4	0	0	0
	Stimulation	10	1,800	5,100	0	264
	Anergy	0	81	150	0	4
pGL10	Rest	0	4	0	0	0
	Stimulation	76	7,600	14,000	0	360
	Anergy	0	90	470	0	74

Th1 clones were incubated overnight with culture media (rest), irradiated APCs plus 250 μg/ml ova peptide (stimulation), or fixed APCs plus 250 μg/ml ova peptide (anergy) and assayed for proliferation by [³H]thymidine incorporation at 48 h. Supernatants were collected at 24 h after stimulation and assayed for lymphokine production as described in Materials and Methods.

and IFN-γ, but little or no proliferation, and no detectable IL-2 (Table 1). Furthermore, as has been previously documented for other Th1 clones (2, 13–15), antigen presentation by fixed APC induced a state of anergy in the T cells resulting in an inability to proliferate or produce IL-2 in response to restimulation with conventional APC at any of the antigen doses tested (Fig. 1). The nonresponsive state in these clones was shown to be long-lived, as the clones could be rested for at least 7–9 d after anergy induction without regaining the ability to respond to antigenic stimulation. The anergized cells remain viable throughout this rest period and will proliferate equivalently to normal resting T cells in response to exogenous IL-2 (data not shown). In contrast, both resting cells and cells that had been stimulated with irradiated APCs did respond to antigen in a dose-dependent manner via proliferation and lymphokine production, although in some experiments (see Fig. 1 *A*), the activated cells were still proliferating from the previous stimulation. Thus, antigen presentation by fixed APC results in TCR engagement in the absence of accessory function as evidenced by the stimulation of some lymphokine production without proliferation, and the induction of a long-lived anergic state.

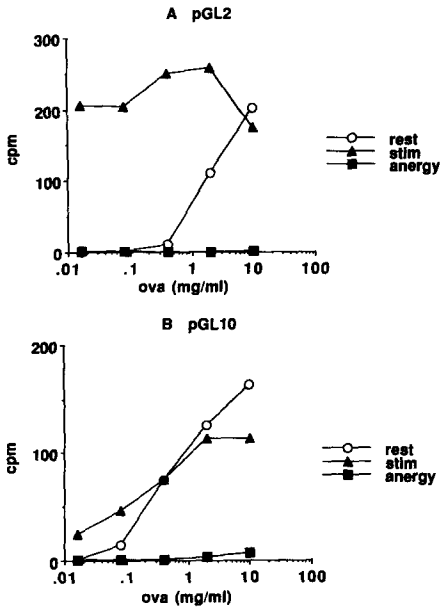


Figure 1. Induction of nonresponsiveness of Th1 clones by preincubation with fixed APCs plus peptide antigen. T cells were incubated overnight with culture media alone (rest), irradiated APC plus antigen (stim), or fixed APC plus antigen (anergy), purified from APC, and restimulated with irradiated APC plus antigen. Data from two independent Th1 clones, pGL2 (*A*) and pGL10 (*B*), are cpm (×10³) after [³H]thymidine incorporation at 48 h. The high level of proliferation of the stimulated T cells at low antigen concentration is due to continued proliferation from the previous stimulation. cpm of thymidine incorporation in the absence of antigen for stimulated pGL2 was 172,185 ± 1.63, and for stimulated pGL10 was 32,401 ± 37.51.

Triggering through the TCR in the Presence of Accessory Function Upregulates the Nuclear Factors That Regulate IL-2 Transcription. The IL-2 promoter contains several highly conserved regions that have been shown to bind transcription factors that regulate the level of IL-2 expression (Fig. 2). Much of the data describing the IL-2 promoter and the transcription factors that regulate it have come from the human Jurkat (19, 21, 23, 24) and mouse EL4 (20, 22) T cell tumor lines. Recently, Granelli-Piperno et al. have shown that these same factors are also present in activated human peripheral T lymphocytes (25, 26). To verify that the IL-2 gene was being transcriptionally regulated similarly in our T cell clones, pGL2 and pGL10 were stimulated for 16 h with irradiated APCs, plus peptide antigen and nuclear extracts were isolated from the T cells after purification from the APCs. These extracts were then assayed by gel mobility shift for the presence of NF-AT, AP-1, Octomer, and NF-κB binding factors (Fig. 3). Indeed, these normal T cell clones induced the same overall pattern of transcription factors after antigen presentation by irradiated splenocytes as has been observed for EL4 and Jurkat tumor cell lines stimulated by mitogens (19, 20).

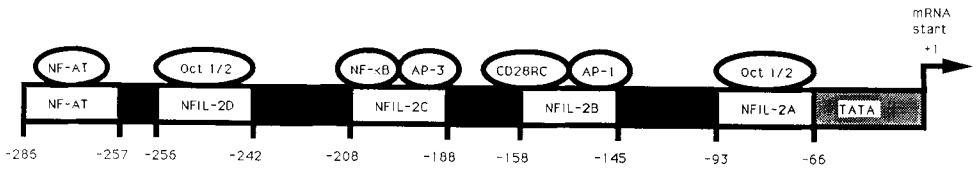


Figure 2. IL-2 enhancer with known protein binding sites. Putative DNA binding proteins are noted above binding sites (19, 21–24, 32, 56).

Specifically, NF-AT is significantly upregulated over resting levels of little or no NF-AT. In addition, the AP-1 protein is induced to high levels after activation. Although in some experiments, the AP-1 protein appeared as two forms on the gel mobility shift assay, it was consistently upregulated upon both types of stimulation, and there was no preferential induction of either form in the stimulated nuclear extracts. The Oct-1 protein generally stayed at ubiquitous resting levels, but in some experiments, there was a slight induction of this nuclear factor (Fig. 3 *A*). The Oct-2 protein, which is normally present in B cells and is important in Ig gene regulation (40, 41, 42), was induced slightly in the pGL2 cell line, and more fully in the pGL10 cell line. The induction of Oct-2 was not seen in the stimulated T cell tumor lines (19, 21–24) and was slightly induced in the peripheral T lymphocytes (25, 26), and may be an important difference between the T cell clones and the tumor lines. For NF-κB binding factors, we identify at least two forms of this factor present in activated cells using the consensus NF-κB probe. There is a slower migrating form that we refer to as B1 (see Fig. 3) that is

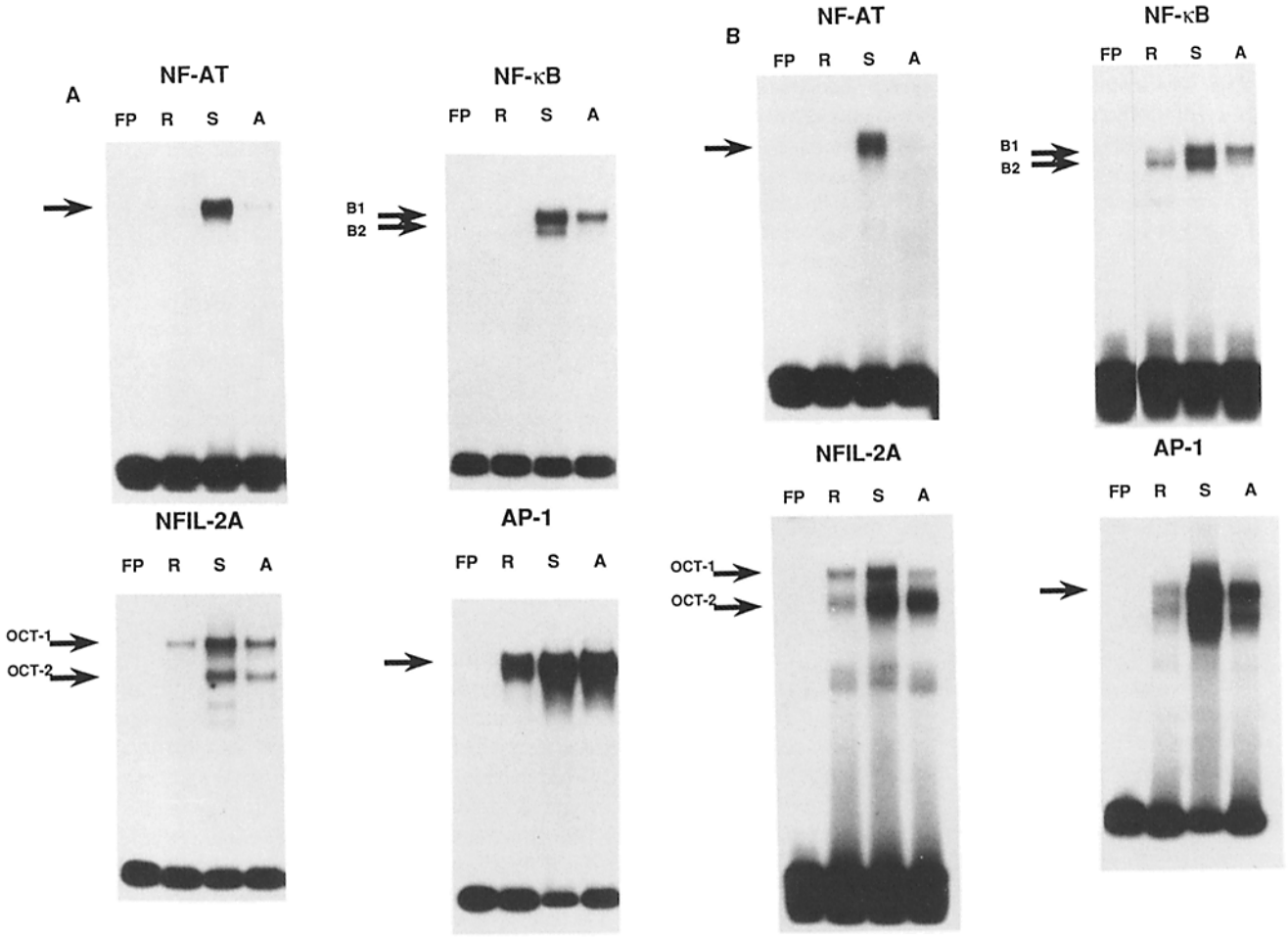


Figure 3. Failure to induce NF-AT and one form of NF-κB in T cell clones stimulated with fixed APCs and peptide antigen. pGL2 (*A*) and pGL10 (*B*) were stimulated overnight with irradiated or fixed APCs plus 250 μg/ml OVA peptide, purified from the APCs, and nuclear extracts were made. Gel mobility shift assays were performed on these extracts with the following probes, NF-AT, NF-κB, NFIL-2A, and AP-1. Consensus sites for NF-kβ and AP-1 were used as probes because several proteins have been reported to bind to the NFIL-2C and NFIL-2B regions and we were interested in looking specifically at NF-kB and AP-1 binding proteins in these cells (19, 20). (*FP*) Free probe; (*R*) incubation in culture media; (*S*) incubation with irradiated APC plus OVA peptide; (*A*) incubation with fixed APC plus OVA peptide. (→) Position of gel shifted complexes.

sometimes present in resting cells, and that is consistently induced to high levels during activation. There is also a faster migrating form that we refer to as B2 that is also upregulated during activation, but at slightly lower levels than is B1.

Engagement of the TCR in the Absence of Accessory Function Fails to Upregulate Two Nuclear Factors that Are Involved in IL-2 Gene Regulation. To address the molecular mechanism whereby the T cell clones failed to express IL-2 after stimulation with fixed APCs, nuclear extracts from these T cells were examined for expression of the same set of DNA binding proteins assayed above. T cell clones were incubated overnight with fixed APCs and peptide antigen, and analyzed in parallel with the activated cells described above for the expression of NF-AT, AP-1, Octomer, and NF-κB binding proteins by gel mobility shift assays (Fig. 3).

Stimulation by fixed APCs induced a profile of transcription factors in our T cell clones that differed from the fully stimulated cells in two ways. First, little or no NF-AT was induced after engagement of the TCR in the absence of accessory function. NF-AT is a T cell–specific factor (21) that has been shown to be critical for full IL-2 gene induction. In the absence of the distal NF-AT binding region, induction of transcription from the IL-2 promoter after transient transfection into Jurkat cells is ~30% of maximal, and removal of the proximal NF-AT site further reduces the activity of the promoter (31). In addition, recent results by Fiering et al. indicate that there is a threshold level of NF-AT that must be present for transcription to occur (31), suggesting that the small amount of NF-AT produced during fixed APC stimulation in pGL2 cells (Fig. 3 *A*) may not be enough to allow for the transcription of the IL-2 gene.

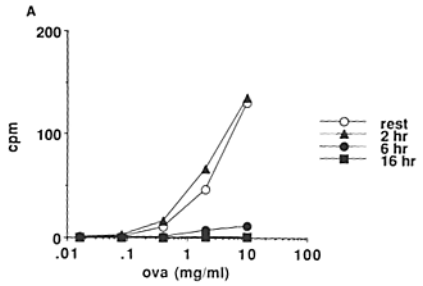
The second observation that may be involved in preventing IL-2 transcription after stimulation with fixed APCs is that the faster migrating form of the NF-κB binding factor, B2, is not induced, while the slower migrating form, B1, is slightly induced. In contrast, both forms are induced in the fully activated state (Fig. 3). Molitor et al. have shown that there is a family of NF-κB proteins made up of different subunits in association with the P55 subunit that are induced after stimulation by PMA in Jurkat T cells (43). The various forms of NF-κβ have been shown to bind with different affinities to different promoter regions that contain NF-κB-like sequences. However, it is not known if these two forms function differently in transcriptional regulation.

It is interesting that the rest of the nuclear factors we examined were induced both with TCR occupancy alone, and in combination with accessory signals (Fig. 3). Oct-1 levels generally remained at resting levels and Oct-2 was present at high levels under both stimulation conditions. Since the octomer proteins are involved in the regulation of many other genes in the cell, they may be less likely to be influenced with TCR signalling. In addition, AP-1 was also induced to similar levels under both conditions of T cell stimulation. In some experiments, however, the level of AP-1 produced was slightly less with TCR triggering in the absence of accessory function as compared with complete stimulation, but was still induced significantly over resting levels (see pGL10 in Fig. 3 *B*). Although the levels of octomer binding proteins and of AP-1 in T cells stimulated with fixed APCs can vary slightly from experiment to experiment as compared with T cells stimulated with irradiated APCs, we have consistently observed the failure to fully induce NF-AT and the B2 form of the NF-κB binding protein after stimulation with fixed APC and nominal antigen. Therefore, it appears that the lack of costimulatory molecule engagement prevents the induction of these two transcription factors.

NF-AT and NF-κB-B2 Are Not Induced at Any Time after Stimulation with Fixed APC. It was possible that the reason why NF-AT and NF-κB-B2 were not present in our nuclear extracts at 12–16 hours poststimulation with fixed APC was that these factors had peaked earlier and had already declined to resting levels by the time the cells were harvested. Therefore, T cells were stimulated with fixed APC and peptide antigen for 2, 6, and 16 h and nuclear extracts were made at these times. Proliferative anergy was induced by 6 h of stimulation with fixed APCs (Fig. 4 *A*), providing evidence that TCR engagement had occurred. Analysis of nuclear extracts made from these cells showed that both NF-AT and the B2 form of NK-κB were induced minimally, if at all, at any time after stimulation with fixed APC. In contrast, the B1 form of NF-κB and AP-1 were induced maximally at 6 h after stimulation (Fig. 4 *B*). Thus, the lack of induction of NF-AT and NK-κB-B2 throughout the entire 16 h after antigen presentation by fixed APC may account for the absence of IL-2 gene expression.

Restimulation of Nonresponsive Cells Induces the Same Pattern of Transcription Factor Expression Associated with Normal T Cell Activation, but Does Not Result in IL-2 Gene Expression. Once anergized, Th1 clones will remain viable for extended periods of time (up to 7–9 d with pGL2 and pGL10) and will proliferate to exogenous IL-2. However, when restimulated with normal APCs, these cells will not express any detectable IL-2 and will not proliferate (see Fig. 1). To determine whether the failure to express NF-AT and/or the B2 form of the NF-κB binding factor was permanent in anergized cells, we examined the expression of these factors after restimulation of anergic cells with antigen and conventional APCs (Fig. 5). Specifically, T cells were induced to a nonresponsive state by incubation overnight with fixed APCs and peptide antigen, and then purified from the APCs the next day. The nonresponsive T cells were subsequently rested in culture media for 2 d. At this time, an aliquot of anergized cells were harvested, and restimulated by incubation for 16 h with irradiated APCs and peptide antigen. Nuclear extracts were made from these anergized and restimulated cells (ReS), from anergized cells that were not stimulated (A'), from completely rested cells (R), and from rested cells that had been stimulated with irradiated APCs and antigen (S). The extracts were examined for pertinent transcription factors by gel mobility shift assay.

An aliquot of the cells used to make nuclear extracts were tested to confirm that anergy had been induced in these cells (Fig. 5 *B*). When restimulated with irradiated APC and antigen, the anergized cells failed to synthesize detectable levels



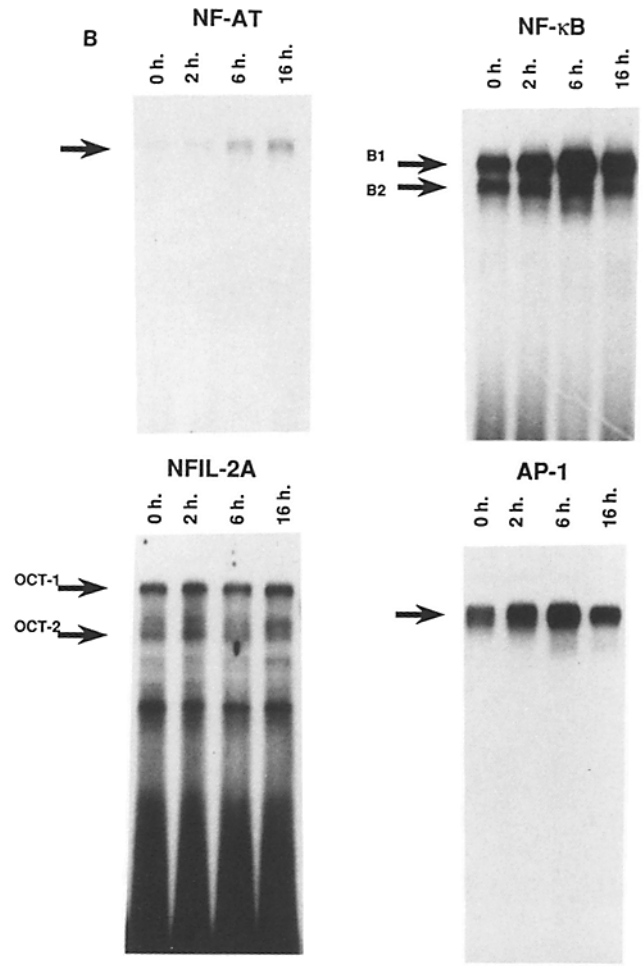


Figure 4. Kinetics of anergy induction and transcription factor expression after TCR occupancy in the absence of accessory function. pGL2 cells were treated for 0, 2, 6, and 16 h with fixed APC and peptide antigen. The T cells were purified and analyzed for proliferative anergy (*A*) as described in Fig. 1 and for the induction of transcription factors (*B*) by gel mobility shift assays as described in Fig. 3.

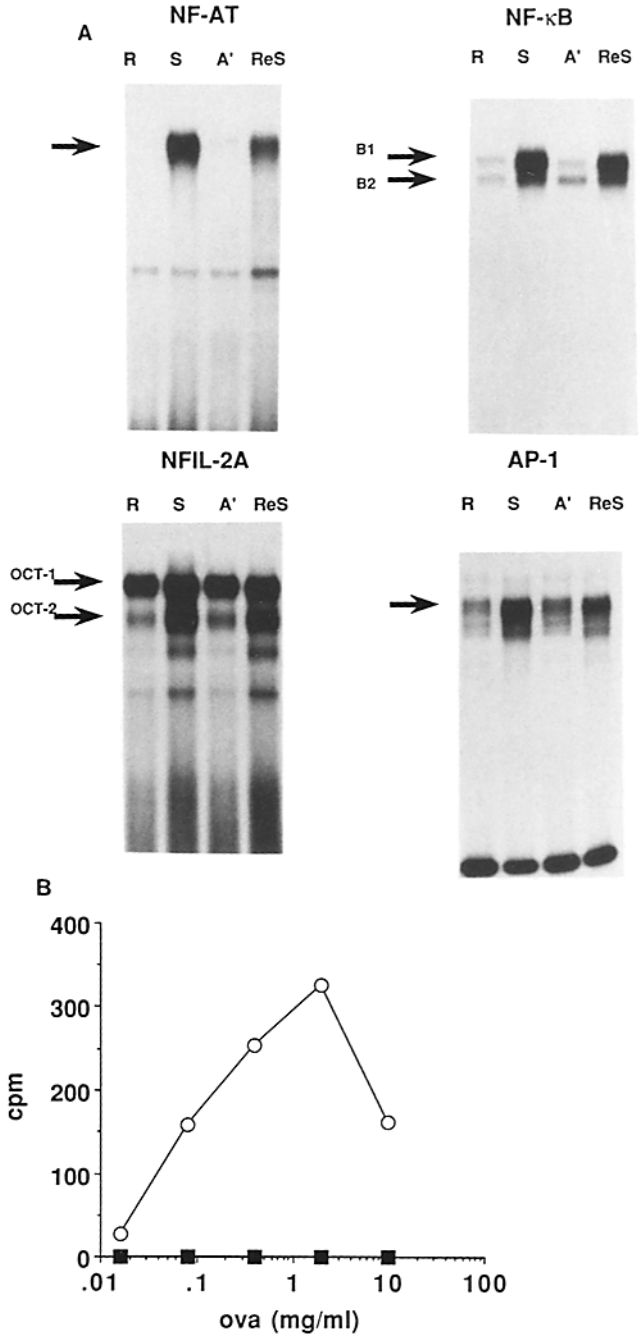


Figure 5. Anergized T cells induce the same set of transcription factors as resting cells, but fail to proliferate or produce IL-2, when restimulated with irradiated APC and antigen. (*A*) Gel mobility shift assays of pGL2 cells. T cells were rested in culture media (*R*), or incubated with irradiated APC plus OVA peptide for 16 h (*S*) as described in Fig. 3. In addition, T cells were anergized by incubation of resting cells with fixed APC plus OVA peptide overnight, purified from the APCs, and rested in culture media for 2 d. Nuclear extracts were made from these cells after the 2 d rest period (*A'*). An aliquot of these same cells were restimulated by incubation with irradiated APC plus OVA peptide for 16 h and nuclear extracts were isolated (*ReS*). Gel mobility shift assays were performed as described in Fig. 3. (*B*) Proliferative response of anergized pGL2 from Fig. 5 *A* above. An aliquot of the cells, R and A', used to make nuclear extracts above were incubated with irradiated APC and antigen and tested for proliferation as described in Fig. 1. Data are cpm × 10^3 after [^{3}H]thymidine incorporation at 48 h.

of IL-2 (data not shown), and to proliferate. Surprisingly, although functional anergy had been achieved (Fig. 5 *B*), all of the nuclear factors associated with IL-2 gene expression, Oct-1, Oct-2, AP-1, NF-κB-B1, NF-κB-B2, and NF-AT, were induced similarly in both resting and anergized cells that had been stimulated in the same manner (Fig. 5 *A*). More specifically, the levels of transcription factor expression in anergized cells that had been rested in culture media for 2 d generally declined back to resting levels, and were comparably induced

after restimulation. NF-AT levels for restimulated cells in this experiment did not increase to quite the same levels as that seen after stimulation of resting cells, but in other experiments, did reach the same high levels seen in stimulated cells. In addition, in this experiment, the B2 form of NF-κB appears to be slightly higher in rested anergized cells as compared with rested cells and may be due to a minor fluctuation in basal levels of this factor over time in anergized cells. We have seen slight differences in resting levels of the various transcription factors in our clone in various experiments and this may depend on the length of time the clone has been rested since the last stimulation. However, in all experiments, we see a comparable induction of all the transcription factors in both stimulated rested cells and in restimulated anergized cells. Thus, the failure to induce IL-2 gene expression during anergy induction and during restimulation of anergized cells appears to be controlled by independent mechanisms. These results suggest that there may be additional mechanisms that regulate IL-2 gene expression during restimulation of nonresponsive T cells.

Discussion

In this study, we have shown that the failure to express IL-2 in T cell clones after antigen presentation by fixed APC can be attributed to the lack of upregulation of the transcription factors NF-AT, and the slower migrating form of the NF-κB binding protein, B2. The lack of these factors may fail to allow for the assembly of a complete transcriptional complex on the IL-2 promoter, in the absence of which the IL-2 gene cannot be expressed. In contrast, stimulation of the T cells by irradiated APC, a situation that allows for the engagement of costimulatory molecules, upregulates the expression of the transcription factors important in inducing transcription of the IL-2 gene. Thus, costimulation appears to function directly with TCR engagement in the transcriptional regulation of the IL-2 gene in responding T cells. It is interesting that although other lymphokines, such as IL-3 and IFN-γ, can be induced by signalling only through the TCR, their expression can be greatly enhanced in the presence of costimulatory activity. The promoter regions of these lymphokines, as well as many others (44), contain an NF-κB consensus sequence. Thus, the lower levels of lymphokine expression, including IL-2, -3, and IFN-γ, after stimulation in the absence of accessory function may be attributed to the failure to induce the NF-κB-B2 form, and the complete absence of IL-2 expression may result from the failure to express NF-AT.

The failure to induce either NF-AT or the NF-κB-B2 binding protein may be explained by several reasons. First, these factors themselves may be under transcriptional and/or translational control, and in the absence of a costimulatory signal, they are not synthesized. Second, there may be an inhibitor that is pre-existing or newly synthesized, which prevents translocation of these factors to the nucleus or alters the protein in some way as to render it transcriptionally inactive. The inhibitor for NF-κB, IκB, has been described by Baeuerle and Baltimore (45) as binding to the NF-κB protein in the cytoplasm and releasing it to the nucleus upon T cell activation with mitogens. There has not been an inhibitor described for NF-AT. We do not feel, however, that the failure to express these factors during anergy induction results from a decrease in the ability of fixed APC to trigger the TCR. We know that the TCR has been engaged with fixed APCs, because the cells will express some lymphokines, and these cells have been functionally anergized. In addition, our preliminary results indicate that when we compensate for the decrease in the efficiency of antigen presentation by fixed cells by adjusting the concentration of antigen used, NF-AT and NF-κB-B2 are still induced significantly greater after presentation by irradiated APC than by fixed APC.

There are two situations in which we have found that the levels of NF-AT can be varied experimentally with TCR occupancy alone. In the first situation, the TCR is occupied not by its specific ligand, but by a mAb to the CD3ε chain of the TCR, 145-2C11 (46). At high doses, the anti-CD3 mAb can cause a nonresponsive state to be induced in T cell clones, similar to the state induced by fixed APCs and antigen. At these same doses, however, T cells are in a state of high dose suppression whereby the cells fail to proliferate to the anti-CD3 mAb, as well as to exogenous IL-2 (47, 48). In addition, in some Th1 clones, incubation on immobilized anti-CD3 mAb alone can also stimulate these cells to produce IL-2, whereas, TCR engagement by fixed APC and peptide antigen does not (11, 48). When nuclear extracts were examined from cells stimulated with anti-CD3 mAb, we saw the induction of significant but suboptimal levels of NF-AT, with all other factors, including NF-κB, being induced similarly when the TCR is engaged by fixed APC and peptide antigen (C. Go and J. Miller, unpublished observations). The observed induction of NF-AT in our T cell clones after stimulation with anti-CD3 mAb is consistent with the observation that some Th1 clones, including the ones examined in this study, can express IL-2 in response to anti-CD3 stimulation alone, but remain nonresponsive to subsequent stimulation. In the second situation, the levels of NF-AT can vary considerably depending on the technique used for isolating nuclear extracts. For example, in our laboratory, the Digham et al. (19) method of purifying nuclear extracts does not preserve NF-AT binding ability, even in stimulated cells. Under other preparative conditions, NF-AT is extracted inefficiently, and we have found that in these cases, apparently similar levels of NF-AT are found in activated and anergized cells. In the data that we have presented here, we have optimized the extraction of NF-AT and we are confident that the in vitro data reflect a real decrease in the amount of NF-AT produced in vivo during TCR occupancy in the absence of accessory function in these T cell clones.

The lack of NF-AT expression after anergy induction is consistent with the model proposed by Granelli-Piperno et al. (26) in human primary T cells. Specifically, they propose that the induction of NF-AT activity requires two signals, namely PMA and PHA, anti-CD3, or anti-CD28. Similarly, in our system, when only one of the signals is given to the T cell clone by stimulation with fixed APC, NF-AT binding

activity is not present. When both signals are given through stimulation with irradiated APC, a high level of NF-AT is induced. Both of these systems fit the hypothesis by Flanagen et al. (49) that there are two subunits of NF-AT that bind together in the nucleus to form a functional DNA binding protein, and each of the signals is important in the formation of this complex.

Although NF-AT plays a major role in the transcriptional control of the IL-2 gene, the lack of the faster migrating form of the NF-κB binding protein, B2, in T cells stimulated by fixed APCs may be equally important to the block in IL-2 expression. The functional role of the two forms of NF-κB, B1 and B2, in transcription have not been elucidated, but it is apparent that different cell types express different levels of each form and various enhancers can bind each form at varying affinities (43). In addition, at least two NF-κB-like binding regions in the IL-2 promoter have now been identified. The first region was identified by in vitro footprinting as the NFIL-2C region (19, 20). Two transcription factors, NF-κB and AP-3, have been reported to bind at this region, and have very similar consensus binding sequences (33, 50). Indeed, the NFIL-2C binding protein can be competed from its binding site by both the consensus sites of NF-κB and AP-3 (data not shown), and it is not clear which of these factors, or which of the NF-κB forms, are footprinting in this region. However, when this site was deleted in IL-2 promoter reporter gene constructs in Jurkat T cells, there was no decrease in expression during activation, as compared with the full length promoter (19). These data suggest that the NFIL-2C site may not be critical to the upregulation of the IL-2 gene. In contrast, Hemar et al. (51) have recently shown that this region is critical in controlling the expression of the IL-2 gene in a T cell line that constitutively expresses IL-2. Analysis of nuclear extracts from these cells reveals two NF-κB binding proteins that gel shift in a pattern that is very similar to that seen in our T cell clones. Hemar et al. (51) suggest that the slower migrating form may be the NF-κB protein composed of the p65-p50 heterodimer, while the faster migrating form may be the KBF-1 protein composed of the p50 subunit. The NF-κB protein has a higher affinity for the Ig κB binding site, whereas the KBF-1 protein has a higher affinity for the NFIL-2C site from the IL-2 promoter. These data suggest that KBF-1 may be more important in IL-2 gene regulation than NF-κB, and when KBF-1 is not induced during stimulation of T cell clones by fixed APCs, IL-2 transcription does not occur.

Engagement by B7 by CD28 recently has been implicated as an important costimulatory interaction in T cell activation (3–7). Triggering of CD28 by antibodies or by transfected cells that express B7 can facilitate stimulation of IL-2 production in conjunction with anti-CD3 antibodies (4–7). Recently two groups have reported that specific nuclear factors that bind to the IL-2 promoter near the AP-1 site can be induced after stimulation of T cells with anti-CD28 antibodies (42, 53). It is interesting that one of these appears to be an NF-κB binding protein, as binding to the CD28 response element in the IL-2 promoter is competed efficiently by the HIV-1 NF-κB site (53). These data are consistent with our finding that in the absence of costimulation, T cells fail to express one of the two NF-κB binding proteins present during normal T cell activation and suggest that the NF-κB-B2 form may be the critical component involved in regulation of the IL-2 gene. Experiments to test whether B7/CD28 engagement can induce one or both of the missing transcription factors are presently underway.

In contrast to the induction phase of anergy, restimulation of previously anergized T cells with conventional APCs, does induce the normal pattern of transcription factors associated with IL-2 expression, although no IL-2 is produced. These data suggest the existence of additional mechanisms to regulate the IL-2 gene. One possibility is that the IL-2 mRNA is posttranscriptionally regulated in anergized T cells, making it extremely unstable after its induction upon restimulation. Another possibility may be that the control functions directly on the transcription factors themselves, such that these factors retain their DNA binding activity, but are modified so that they no longer promote transcriptional activity. Alternatively, a silencer region, such as that recently identified by Williams et al. (54), may be involved in downregulating IL-2 transcription during T cell anergy. It is interesting that this putative silencer region is thought to function during normal T cell activation to shut off IL-2 transcription. The recent results of DeSilva et al. (55), demonstrating that inhibition of T cell proliferation during normal T cell activation can induce T cell anergy, are consistent with a role of negative regulatory factors in T cell anergy. Specifically, during T cell activation, normal proliferation of the T cell may dilute out any negative regulatory factor induced during the stimulation response, allowing the IL-2 gene to return to an activatable state. During anergy induction, the IL-2 gene is not induced, but the repressor(s) is induced normally. The lack of T cell proliferation that results from the failure to express IL-2 leads to the establishment of a stable, nontranscriptionally active IL-2 gene complex. This complex would remain inactive even in the face of activation of the normal panel of positive regulatory elements associated with IL-2 transcription during restimulation of anergized cells. Experiments are in progress to test this model.

We thank Dr. H. Singh for critical review of the manuscript, Dr. F. Fitch for the gift of the Th1 clones, T. Wong for help in performing the lymphokine assays, Dr. J. Bluestone for the mAb, 145-2C11, (all of the University of Chicago) and Dr. G. R. Crabtree (Stanford University, Stanford, CA) for assistance with nuclear extract preparation. Oligonucleotides were provided by the University of Chicago Cancer Center Core Facility.

C. Go was supported by the Medical Scientist Training Program Grant (PHS-GM-07281) and the Arthritis Foundation Medical Student Fellowship. Dr. J. Miller was supported by grants from the National Institutes of Health (GM-42071 and DK-42857) and the Cancer Research Institute.

Address correspondence to Dr. Jim Miller, Dept. of Molecular Genetics and Cell Biology, University of Chicago, 920 E. 58th Street, Chicago, IL 60637.

Received for publication 9 December 1991 and in revised form 14 February 1992.

References

1. Schwartz, R.H. 1985. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu. Rev. Immunol.* 3:237.
2. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445.
3. Linsley, P.S., E.A. Clark, and J.A. Ledbetter. 1990. T-cell antigen CD28 mediates adhesion with B cells by interacting with activation antigen B7/BB-1. *Proc. Natl. Acad. Sci. USA.* 87:5031.
4. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
5. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB-1 provides costimulatory signal for alloactivation of $CD4^+$ T cells. *J. Exp. Med.* 173:759.
6. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B7 provides a costimulatory signal which induces T cells to proliferate and secrete interleukin-2. *Proc. Natl. Acad. Sci. USA.* 88:6575.
7. Jenkins, M.J., P.S. Taylor, S.D. Norton, and K.B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 146:2461.
8. van Seventer, G.A., W. Newman, Y. Shimizu, T.B. Nutman, Y. Tanaka, K.J. Horgan, T.V. Gopal, E. Ennis, D. O'Sullivan, H. Grey, and S. Shaw. 1991. Analysis of T cell stimulation by superantigen plus major histocompatibility complex class II molecules or by CD3 monoclonal antibody: costimulation by purified adhesion ligands VCAM-1, ICAM-1, but not ELAM-1. *J. Exp. Med.* 174:901.
9. Shimizu, Y., G.A. van Seventer, K.J. Horgan, and S. Shaw. 1990. Costimulation of proliferative responses of resting $CD4^+$ T cells by the interaction of VLA-4 and VLA-5 with fibronectin or VLA-6 with laminin. *J. Immunol.* 145:59.
10. Bierer, B.E., A. Peterson, J.C. Gorga, S.H. Herrmann, and S.J. Burakoff. 1988. Synergistic T cell activation via the physiological ligands for CD2 and the T cell receptor. *J. Exp. Med.* 168:1145.
11. Williams, M.E., A.H. Lichtman, and A.K. Abbas. 1990. Anti-CD3 antibody induces unresponsiveness to IL-2 in Th1 clones but not in Th2 clones. *J. Immunol.* 144:1208.
12. Evavold, B.D., and J. Quintans. 1989. Accessory cell function of Th2 clones. *J. Immunol.* 143:1784.
13. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302.
14. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, T.M. Chused, and R.H. Schwartz. 1987. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc. Natl. Acad. Sci. USA.* 34:5409.
15. Quill, H., and R.H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol.* 138:3704.
16. Markmann, J., D. Lo, A. Naji, R.D. Palmiter, R.L. Brinster, and E. Heber-Katz. 1988. Antigen presenting function of class II MHC expressing pancreatic beta cells. *Nature (Lond.).* 336:476.
17. Gaspari, A.A., M.K. Jenkins, and S.I. Katz. 1988. Class II MHC-bearing keratinocytes induce antigen-specific unresponsiveness in hapten-specific Th1 clones. *J. Immunol.* 141:2216.
18. Jenkins, M.K., C. Chen, G. Jung, D.L. Mueller, and R.H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J. Immunol.* 144:16.
19. Durand, D.B., J. 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.
20. Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, R. Mercurio, and M. Karin. 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:465.
21. Shaw, J., P.R. 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 (Wash. DC).* 241:202.
22. Brunvand, M.W., A. Schmidt, and U. Siebenlist. 1989. Nuclear factors interacting with the mitogen-responsive regulatory region of the interleukin-2 gene. *J. Bio. Chem.* 263:18,904.
23. Shibuya, H., and T. Taniguchi. 1989. Identification of multiple cis elements and transacting factors involved in the induced expression of the human IL-2 gene. *Nucleic Acids Res.* 17:9173.
24. Hoyos, B., D.W. Ballard, E. Bohnlein, M. Siekevitz, and W.C. Greene. 1989. Kappa B-specific DNA binding proteins: role in the regulation of human interleukin-2 gene expression. *Science (Wash. DC).* 244:457.
25. Granelli-Piperno, A., P. Nolan, K. Inaba, and R.M. Steinman. 1990. The effect of immunosuppressive agents on the induction of nuclear factors that bind to sites on the interleukin 2 promoter. *J. Exp. Med.* 172:1869.
26. Granelli-Piperno, A., and P. Nolan. 1991. Nuclear transcription factors that bind to elements of the IL-2 promoter. Induction requirements in primary human T cells. *J. Immunol.* 147:2734.
27. Gajewski, T.F., M. Pinnas, T. Wong, and F.W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response

to distinct antigen-presenting cell populations. *J. Immunol.* 146:1750.
28. Shimonkevitz, R., J. Kappler, P. Marrack, and H. Grey. 1983. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J. Exp. Med.* 158:303.
29. Gunter, K.C., T.R. Malek, and E.M. Shevach. 1984. T cell-activating properties of an anti-thy-1 monoclonal antibody. Possible analogy to OKT3/LEU-4. *J. Exp. Med.* 159:716.
30. Kappler, J.W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, *H-2*-restricted, interleukin-2-producing T cell hybridomas. Lack of independent antigen and *H-2* recognition. *J. Exp. Med.* 153:1198.
31. Fiering, S., J.P. Northrop, G.P. Nolan, P.S. Mattila, G.R. Crabtree, and L.A. Herzenberg. 1990. Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. *Genes & Dev.* 4:1823.
32. Lee, W., A. Hasliner, M. Karin, and R. Tijian. 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene. *Nature (Lond.).* 325:368.
33. Pierce, J.W., M. Lenardo, and D. Baltimore. 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. *Proc. Natl. Acad. Sci. USA.* 84:1482.
34. Fried, M., and D.M. Crothers. 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9:6505.
35. Garner, M.M., and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. *Nucleic Acids Res.* 9:3047.
36. Mossman, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65:55.
37. Ihle, J.N., J. Keller, J.S. Greenberger, L. Henderson, R.A. Yetter, and H.C. Morse III. 1982. Phenotypic characteristics of cell lines requiring interleukin 3 for growth. *J. Immunol.* 129:1377.
38. Ihle, J.N., J. Keller, S. Oroszian, L.E. Henderson, T.D. Copeland, F.W. Fitch, M.B. Prystowsky, E. Goldwasser, J.W. Schrader, E. Palaszynzski, M. Dy, and B. Lebel. 1983. Biological properties of homogenous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, B cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. *J. Immunol.* 131:282.
39. Schell, S., D. Nelson, H. Fozzard, and F.W. Fitch. 1987. The inhibitory effects of K^+ channel-blocking agents on T lymphocyte proliferation and lymphokine production are "non-specific". *J. Immunol.* 139:3224.
40. Scheideriet, C., A. Heguy, and R.G. Roeder. 1987. Identification and purification of a human lymphoid-specific octamer-binding protein (OTF-2) that activates transcription of an immunoglobulin promoter in vitro. *Cell.* 51:783.
41. Bergman, T., B. Strich, H. Sharir, R. Ber, and R. Laskov. 1990. Extinction of Ig gene expression in myeloma × fibroblast somatic cell hybrids is accompanied by repression of the oct-2 gene encoding a B-cell specific transcription factor. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:849.
42. Junker, S., S. Pedersen, E. Schreiber, and P. Matthias. 1990. Extinction of an immunoglobulin κ promoter in cell hybrids is mediated by the octamer motif and correlates with suppression of Oct-2 expression. *Cell.* 61:467.
43. Molitor, J.A., W.H. Walker, S. Doerre, D.W. Ballard, and W.C. Greene. 1990. NF-κB: A family of inducible and differentially expressed enhancer-binding proteins in human T cells. *Proc. Natl. Acad. Sci. USA.* 87:10028.
44. Arai, K., F. Lee, A. Miyajima, S. Miyatake, N. Arai, and T. Yokota. 1990. Cytokines: coordinators of immune and inflammatory responses. *Annu. Rev. Biochem.* 59:783.
45. Baeuerle, P.A., and D. Baltimore. 1988. IκB: A specific inhibitor of the NF-κB transcription factor. *Science (Wash. DC).* 242:540.
46. Leo, O., M. Foo, D.H. Sachs, L.E. Samelson, and J.A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc. Natl. Acad. Sci. USA.* 84: 1374.
47. Nau, G.J., R.L. Moldwin, D.W. Lancki, D. Kim, and F.W. Fitch. 1987. Inhibition of IL-2 driven proliferation of murine T lymphocyte clones by supraoptiman levels of immobilized anti-T cell receptor monoclonal antibody. *J. Immunol.* 139:114.
48. Williams, I.R., and E.R. Unanue. 1990. Costimulatory requirements of murine Th1 clones: the role of accessory cell-derived signals in responses to anti-CD3 antibody. *J. Immunol.* 145:85.
49. Flanagan, W.M., B. Corthesy, R.J. Bram, and G.R. Crabtree. 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature (Lond.).* 352:803.
50. Mitchell, P.J., C. Wang, and R. Tijian. 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell.* 50:847.
51. Hemar, A., S. Cereghini, V. Cornet, V. Blank, A. Israel, W.C. Greene, and A. Doutry-Varsat. 1991. κB binding proteins are constitutively expressed in an IL-2 autocrine human T cell line. *J. Immunol.* 146:2409.
52. 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 (Wash. DC).* 251:313.
53. 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.
54. Williams, T.M., D. Moolten, J. Burlein, J. Romano, R. Bhaerman, A. Godillot, M. Mellon, F.J. Rauscher III, and J.A. Kant. 1991. Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science (Wash. DC).* 254:1791.
55. DeSilva, D.R., K.B. Urdahl, and M.K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J. Immunol.* 147:3261.
56. Lenardo, M.J., A. Kuang, A. Gifford, and D. Baltimore. 1988. NF-κB protein purification from bovine spleen: nucleotide stimulation and binding site specificity. *Proc. Natl. Acad. Sci. USA.* 85:8825.