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CD28 Mediates Transcriptional Upregulation of the Interleukin-2 (IL-2) Promoter through a Composite Element Containing the CD28RE and NF-IL-2B AP-1 Sites

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Mutagenesis studies have demonstrated the requirement for the CD28-responsive element (CD28RE) within the interleukin-2 (IL-2) promoter for transcriptional upregulation by CD28. Here, we demonstrate that CD28 responsiveness is conferred by a composite element containing both the CD28RE and the NF-IL-2B AP-1 sites (RE/AP). Mutations at either site within the RE/AP composite element abolish activity. The RE/AP composite element is a site for signal integration within the IL-2 promoter, since its activation is dependent on at least two separate signalling pathways being activated, through the T-cell receptor, CD28, and/or phorbol myristate acetate. Activation is maximal when all three signals occur simultaneously. By using a panel of CD28 cytoplasmic domain mutants, it was found that the transcriptional activation of the RE/AP composite element correlates exactly with the pattern of IL-2 secretion induced by these mutants upon stimulation. Similar to the upregulation of IL-2 secretion, the transcriptional upregulation of the RE/AP composite element by CD28 is FK506 insensitive. The pattern of activation of the RE/AP composite element is different from that observed for either an NFAT or consensus AP-1 site, implying that RE/AP represents a unique element. Using gel shift analysis, we demonstrate that stimulation by CD28 induces the association of the NF-κB family member c-Rel to the CD28RE within the RE/AP composite element. The transcriptional upregulation of IL-2 by CD28 appears, therefore, to be mediated through the RE/AP composite element, involving the association of c-Rel with the CD28RE.

Activation of T cells by recognition of antigens and major histocompatibility complex products on the surfaces of antigen-presenting cells initiates a series of biochemical events leading to T-cell proliferation and cytokine secretion (33). Stimulation through the T-cell receptor (TCR) alone is insufficient for T-cell proliferation or interleukin-2 (IL-2) production. In the absence of costimulation, a state of unresponsiveness, termed anergy, may develop (13, 18). The failures to produce and respond to IL-2 are the major determinants of anergy. CD28, a 44-kDa glycoprotein expressed as a homodimer on most T cells, can mediate costimulation. Induction of anergy in T-cell clones was found to be blocked by activation of CD28 by monoclonal antibodies (7).

One consequence of T-cell activation is the expression of IL-2, due to both transcriptional upregulation and stabilization of IL-2 mRNA. Transcriptional upregulation is mediated by the IL-2 promoter, contained within approximately 300 bp upstream from the transcriptional start site (reviewed in references 11 and 22). Engagement of CD28 enhances activation of the IL-2 promoter resulting from either TCR stimulation or use of phorbol esters such as phorbol myristate acetate (PMA). Mutagenesis studies have demonstrated that a CD28-responsive element (CD28RE), located within the IL-2 promoter between −160 and −152 relative to the transcriptional start site, is required for CD28-induced IL-2 promoter upregulation (3). Similar sites for CD28-mediated transcriptional activation have been discovered within the promoters of the genes encoding IL-3, granulocyte-macrophage colony-stimulating factor, and gamma interferon (4). However, IL-2 production appears to be more tightly dependent upon CD28 costimulation (13).

While the CD28RE is required for CD28-mediated transcriptional activation of the IL-2 promoter, the transcription factors involved in this upregulation remain in question. The CD28RE has homology to a consensus NF-κB site, and the NF-κB family members p50 (NFKB1), p65 (RelA), and c-Rel have been implicated in activation through the CD28RE (5, 17). Additionally, nuclear expression of all three family members is increased upon CD28 stimulation (5). There is also evidence that NFAT, a protein with weak homology to the rel DNA binding domain of the NF-κB family, may also function at the CD28RE (6, 24). The analysis of c-Rel knockout mice has also demonstrated that c-Rel is critical for IL-2 gene expression, including CD28-mediated upregulation (16). However, whether c-Rel plays a direct or an indirect role in IL-2 activation is not yet known. Recent evidence has implicated c-Rel in upregulating AP-1 activity (28) and in a possible functional association with JNK1 (19). In contrast, other studies have also demonstrated the binding to the CD28RE of proteins that do not appear to be of the appropriate molecular weight to belong to either the NF-κB or the NFAT family (1, 4). Therefore, there is still much uncertainty as to how CD28 mediates transcriptional regulation through the CD28RE.

CD28 has also been shown to upregulate the activity of another transcription factor in T cells, AP-1 (23, 30). There are several nonconsensus sites for AP-1 binding within the IL-2 promoter: by itself at the NF-IL-2B site, in association with NFAT proteins at the NF-IL-2E site, and in association with

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octamer proteins at the NF-IL-2A site. In T cells, JNK kinase activity (and therefore activation of AP-1 through one component, c-jun) could not be stimulated through the TCR alone but required costimulatory signals that could be provided by CD28 stimulation (30). Hence, CD28 may transcriptionally activate genes through AP-1 sites in addition to the CD28RE.

Interestingly, the CD28RE of the IL-2 promoter is separated from an adjacent AP-1 site (the NF-IL-2B site) by only 2 bp. Therefore, it seemed possible that full CD28 responsiveness in the IL-2 promoter might be mediated by both of these elements and that they might act synergistically in mediating CD28 upregulation of a reporter construct containing both sites. Our results demonstrate that an element composed of both the CD28RE and the NF-IL-2B AP-1 sites (RE/AP) functions as a composite unit. Mutation at either the CD28RE or the NF-IL-2B AP-1 site abolished all activation, including CD28 upregulation. The RE/AP composite element is a site for signal integration within the IL-2 promoter, since its activation is dependent on at least two separate signalling pathways being activated, i.e., through the TCR, CD28, and/or PMA. Activation is maximal when all three signals occur simultaneously. CD28-mediated activation of the RE/AP reporter is resistant to FK506 inhibition, consistent with CD28 costimulation of IL-2 production being FK506 insensitive. The pattern of activation of the RE/AP composite element is different than that observed with either an NFAT or consensus AP-1 site, implying that RE/AP represents a unique element. By testing with a panel of CD28 cytoplasmic domain mutants, the transcriptional activation of the RE/AP composite element was found to correlate exactly with the pattern of IL-2 secretion induced by these mutants upon stimulation. By gel shift analysis, we demonstrate that stimulation by CD28 induces the association of the NF-κB family member c-Rel with the CD28RE within the RE/AP composite element. Thus, CD28 activation of the IL-2 promoter appears to be mediated by a composite element composed of both the CD28RE and the adjacent NF-IL-2B sites, and c-Rel may be the major component mediating activation of the CD28RE.

MATERIALS AND METHODS

Plasmids. pCMV4 p65 and pCMV4 c-Rel were provided by Warner Greene. pCMV p50 was kindly provided by Tim Finco and Al Baldwin. The RE/AP reporter plasmids were constructed by multimerizing oligonucleotides into the *Sal*I site of pΔODLO, a generous gift from Barry Starr and Keith Yamamoto. The 3xNFAT luc and 4xAP-1 luc reporters have been previously described (28). Each of the RE/AP reporter constructs contains four copies of the oligonucleotide oriented in the reverse direction, as confirmed by sequencing. The oligonucleotides used are as follows (uppercase letters refer to nucleotides from the IL-2 promoter, while lowercase letters denote *Sal*I linkers added for multimerization):

RE/AP,

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5′ tcgagTTTAAAGAAATTCCAAAGAGTCATCAg     3′
3′     cAAATTTCTTTAAGGTTTCTCAGTAGTcagct 5′
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RE_m/AP,

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5′ tcgagTTTAAAGACCTCGAAAAGAGTCATCAg     3′
3′     cAAATTTCTGGAGCTTTTCTCAGTAGTcagct 5′
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RE/AP_m,

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5′ tcgagTTTAAAGAAATTCCAAATCAACATCAg     3′
3′     cAAATTTCTTTAAGGTTTAGTTGTAGTcagct 5′
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RE_m/AP_m,

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5′ tcgagTTTAAAGACCTCGAAAAGCAACATCAg     3′
3′     cAAATTTCTGGAGCTTTTCGTTGTAGTcagct 5′
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Transfections and luciferase assays. Luciferase assays and transfections were performed as previously described (28).

Stimulations. Jurkat or EL-4 T cells were stimulated for either 6 h for luciferase assays or 4 h for generating stimulated nuclear extracts. PMA (Calbiochem) was used at either 1, 5, or 25 ng/ml as described in Results. Ionomycin (Calbiochem) was used at 1 μM. TCR stimulations were performed with the anticlonotypic monoclonal antibody C305, at a 1:1,000 dilution of ascitic fluid. For stimulations for nuclear extracts, C305 was used in solution. For stimulations for luciferase, C305 was bound to the plate. The plates were coated with 10 μg of anti-mouse immunoglobulin in 0.1 M $NaHCO_3$ overnight at 4°C. The plates were washed four times with phosphate-buffered saline (PBS) and incubated with a 1:1,000 dilution of C305 ascitic fluid in PBS overnight at 4°C. Prior to use, the plates were washed four times with PBS. The anti-human CD28 monoclonal antibody (9.3) was a generous gift from the Bristol-Myers-Squibb Pharmaceutical Research Division. It was used at a 1:1,000 dilution of ascitic fluid. The anti-mouse CD28 monoclonal antibody (37.51) was a generous gift from Jim Allison. It was used at a 1:1,000 dilution of 1-mg/ml RPMI 1640.

Radiolabelling oligonucleotides. One hundred nanograms of annealed oligonucleotide was incubated with 10 mM Tris HCl (pH 7.5)–10 mM $MgCl_2$–50 mM NaCl–1 mM dithioerythreitol–1 mM dGTP–1 mM dTTP–5 μCi of [^{32}P]dATP–5 μCi of [^{32}P]dCTP–10 U of Klenow fragment (Boehringer Mannheim) at room temperature for at least 3 h. Free label was removed with a Sephadex G-50 microspin column (Pharmacia).

Electromobility shift assays (EMSAs). Nuclear extracts from Jurkat cells were generated by the method of Schreiber et al. (25), as previously described. The same oligonucleotides used to generate the RE/AP reporter plasmids were used as probes. The NF-κB and AP-1 probe sequences are described elsewhere (28). Gel shifts were performed as previously described, with the following buffer for the reactions: 10 mM HEPES-KOH (pH 7.9), 50 mM NaCl, 5 mM Tris HCl (pH 7.5), 15 mM EDTA, 1 mM dithiothreitol, and 10% glycerol. Two and one-half micrograms of poly(dI-dC) · poly(dI-dC) was used in each reaction as a nonspecific competitor (Pharmacia). Five micrograms of nuclear extract was used in each reaction. Approximately 0.2 ng of radiolabelled probe was used per reaction. Reaction mixtures were incubated for 20 min at room temperature before separation on a 4% nondenaturing 0.5× Tris-borate-EDTA–acrylamide gel. For supershift studies, 1 μl of normal rabbit serum (NRS) or a specific antiserum was added to the extracts 5 min prior to addition of the probe. Anti-p50 (sc-1191x) and anti-p65 (sc372x) antibodies were purchased from Santa Cruz Biotechnology. Anti-c-Rel (1136) antiserum was a generous gift from Nancy Rice.

RESULTS

CD28 costimulation of the IL-2 promoter through the CD28RE (2, 3) or of a consensus AP-1 reporter alone (23, 30) has been previously demonstrated. The CD28RE and adjacent NF-IL-2B AP-1 sites in the IL-2 promoter are separated by only two nucleotides; therefore, it seemed possible that CD28 responsiveness in the IL-2 promoter might be mediated by both of these elements. This could explain the more stringent requirement for IL-2 transcription activation, as compared to other CD28-responsive lymphokines which do not have AP-1 sites adjacent to their CD28REs (4, 13, 32). Thus, these two adjacent elements might act synergistically in mediating CD28 upregulation of a reporter construct containing both sites. An oligonucleotide containing both the CD28RE and the NF-IL-2B AP-1 sites was multimerized to generate a 4xRE/AP luciferase reporter construct (Fig. 1A). In addition, reporter constructs with mutations in either the CD28RE, the NF-IL-2B AP-1 site, or both were created (12, 17). These reporters were subsequently transfected into Jurkat T cells and examined for activation in response to a combination of PMA-ionomycin and, for further activation, by the addition of anti-CD28 antibodies. As shown in Fig. 1B, the wild-type 4xRE/AP reporter construct was activated by PMA-ionomycin and was further upregulated by the addition of anti-CD28 antibodies. Mutations in either the CD28RE or the NF-IL-2B AP-1 site abrogated responses to both stimuli, demonstrating that both of these sites are required for transcriptional activation. This result is surprising since CD28 would be expected to upregulate each site separately. Given this strong interdependence between these two sites for activation and for upregulation by CD28, the combination of the CD28RE and NF-IL-2B AP-1 sites appears to function as a composite element.

The signals required for activation of the composite RE/AP element were further investigated by using Jurkat T cells (Fig. 2). Stimulation with PMA alone, ionomycin alone, anti-CD28 alone, or anti-TCR alone was insufficient to activate RE/AP.

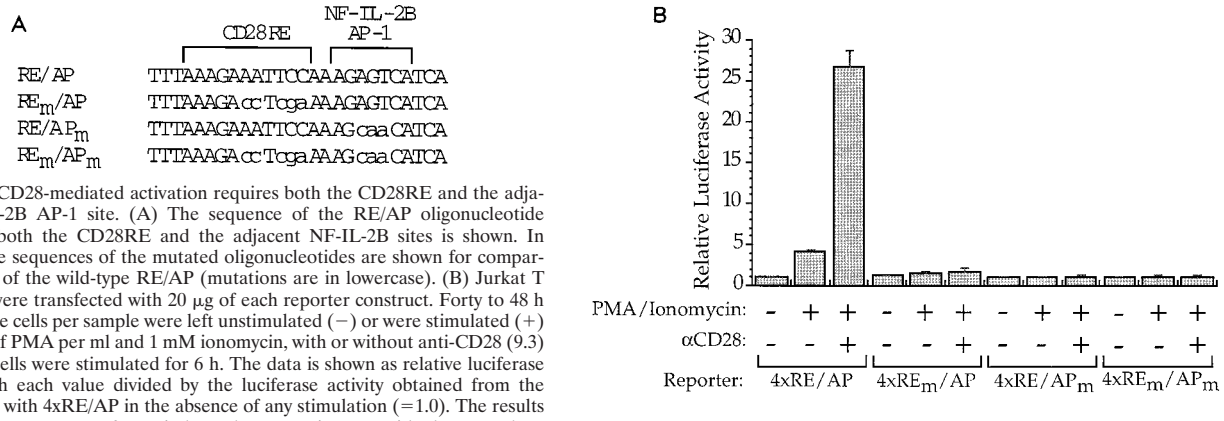

FIG. 1. CD28-mediated activation requires both the CD28RE and the adjacent NF-IL-2B AP-1 site. (A) The sequence of the RE/AP oligonucleotide containing both the CD28RE and the adjacent NF-IL-2B sites is shown. In addition, the sequences of the mutated oligonucleotides are shown for comparison to that of the wild-type RE/AP (mutations are in lowercase). (B) Jurkat T cells (10^7) were transfected with 20 μg of each reporter construct. Forty to 48 h later, 10^5 live cells per sample were left unstimulated (−) or were stimulated (+) with 25 ng of PMA per ml and 1 mM ionomycin, with or without anti-CD28 (9.3) (αCD28). Cells were stimulated for 6 h. The data is shown as relative luciferase activity, with each value divided by the luciferase activity obtained from the transfection with 4xRE/AP in the absence of any stimulation (=1.0). The results represent the averages of two independent experiments, with the error bars reflecting the standard errors of the means.

However, combinations of these signals effectively and strongly activated the RE/AP reporter construct. Stimulation with anti-CD28 and PMA weakly stimulated RE/AP activity, i.e., from two- to sevenfold depending upon the PMA concentration (Fig. 2A); the higher the PMA concentration, the greater the upregulation of the RE/AP reporter. The combination of stimulation with anti-CD28 and anti-TCR also weakly stimulated RE/AP activity, by approximately threefold (Fig. 2B). The combination of anti-TCR and PMA activated RE/AP from 3- to 25-fold, depending on PMA concentration (Fig. 2B). The combination of all three signals, those from PMA, anti-CD28, and anti-TCR, had the greatest effect on RE/AP activity, activating the reporter from 75- to 133-fold (Fig. 2B). The specific effect of CD28 upregulation is greatest at lower PMA concentrations, activating the RE/AP reporter by 25-fold. Similar activation was observed with the combination of PMA and ionomycin; activation ranged from 2- to 24-fold, depending on the PMA concentration, while the addition of anti-CD28 further increased activation from 48- to 116-fold (Fig. 2C). This pattern of activation was observed whether soluble or immobilized anti-TCR was used (data not shown). Similarly, for the murine T-cell lymphoma EL-4, the stimulation of the RE/AP reporter in response to PMA and ionomycin was further increased upon anti-CD28 stimulation (Figure 2D). One might have expected that anti-TCR and anti-CD28 signals alone would be sufficient for stimulation of the RE/AP composite element; however, maximal transcriptional activation also required the addition of PMA. Therefore, the RE/AP composite element may function in vivo to integrate signals not only from the TCR and CD28 but also from a third, distinct pathway activated by PMA. Together, these three signals may mimic the complex interactions which occur when a T cell recognizes an antigen on the surface of an antigen-presenting cell (29).

The upregulation of IL-2 secretion by CD28 has been previously shown to be insensitive to FK506, which inhibits the serine/threonine phosphatase calcineurin (reviewed in reference 14). Similarly, if CD28-mediated upregulation of IL-2 is mediated through the RE/AP composite element, then CD28-mediated transcriptional activation of the RE/AP composite element should also be insensitive to FK506. As shown in Fig. 3A, the activation of the RE/AP reporter construct by the combination of PMA and anti-CD28 is not affected by FK506 addition. Therefore, both the PMA and CD28 pathways leading to RE/AP activation are FK506 insensitive. However, the TCR-mediated upregulation of the RE/AP composite element is sensitive to FK506 (Fig. 3A); transcriptional activation by the combination of anti-TCR and PMA is abolished by FK506 treatment. However, in either case, anti-CD28 stimulation upregulated activation by approximately 10-fold. Therefore, similar to the insensitivity of CD28 upregulation of IL-2 production, the CD28-mediated transcriptional upregulation of the RE/AP composite element is FK506 insensitive.

The activation of the RE/AP reporter element involves a unique element, distinct from either a consensus AP-1 site alone or an NFAT element, which is also found within the IL-2 promoter. The NF-IL-2E NFAT site at approximately −280 relative to the transcriptional start site within the IL-2 promoter is composed of both NFAT and AP-1 proteins (reviewed in reference 21). However, as shown in Fig. 3, the activation profiles of reporter constructs for the composite RE/AP element, the consensus AP-1 site, and the NF-IL-2E NFAT sites in response to various stimuli are quite distinct. By comparing Fig. 3A and B, it can be seen that the NFAT reporter is activated by anti-TCR alone, while this is insufficient for RE/AP activation. Also, anti-CD28 treatment markedly upregulates RE/AP but has little effect on NFAT activation. In addition, FK506 abolishes all activation of NFAT but does not affect transcriptional upregulation of RE/AP by PMA and anti-CD28. Likewise, the activation of RE/AP is separate and distinct from the activation of the consensus AP-1 site (compare Fig. 3A and C). Treatment by anti-TCR alone is sufficient to activate the AP-1 reporter construct and is insensitive to FK506 treatment, while treatment by anti-TCR alone is insufficient to activate RE/AP and is sensitive to FK506 treatment. Therefore, the RE/AP composite element is a unique element, unlike either a consensus AP-1 site or an NFAT site.

To examine whether the transcriptional upregulation of the RE/AP composite element mimics the effects of CD28 upon IL-2 production, we utilized a panel of Jurkat stable cell lines which express either the wild-type murine CD28 or various C-terminal truncations (31). The ability of these mutated CD28 molecules to influence IL-2, as previously reported (31), is summarized in Fig. 4A; only the wild-type CD28 and the T1 C-terminal truncation are capable of increasing IL-2 secretion, while further C-terminal truncations (T2, T3, and TL) cannot. Similarly, only the wild-type and T1 murine CD28 molecules increased activation of the composite RE/AP reporter construct upon stimulation with an antibody specific to murine CD28 (37.51) in combination with stimulation by anti-TCR–PMA (Fig. 4B). No activation of the composite RE/AP element was observed with the T2, T3, or TL mutation. However, in each of these cell lines, transcriptional upregulation of the RE/AP composite element was observed when it was costimulated through the endogenous human CD28 molecule by using

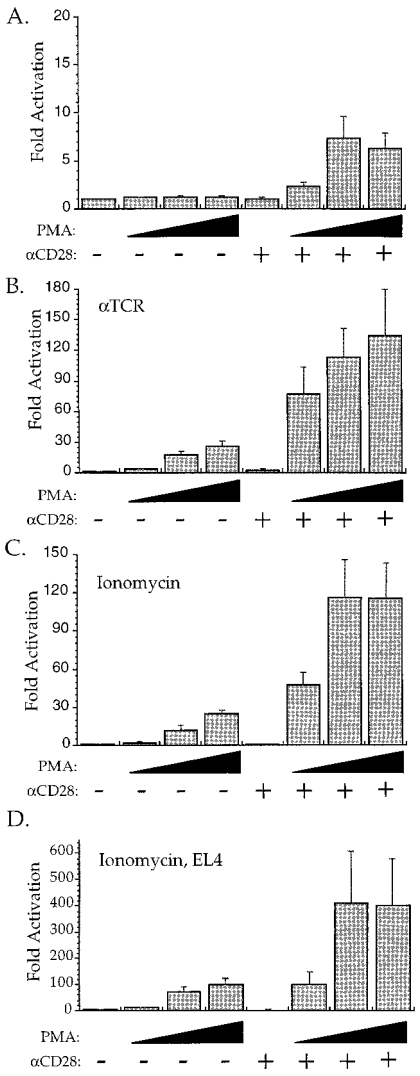

FIG. 2. Optimal stimulation of 4xRE/AP occurs when a combination of stimuli are used. (A) Jurkat T cells (10^7) were transfected with 20 μg of 4xRE/AP. Forty to 48 h later, 10^5 live cells per sample were stimulated with either 1, 5, or 25 ng of PMA per ml and/or anti-CD28 (9.3) (αCD28) as indicated below the graph. Cells were stimulated for 6 h. The data is shown as fold activation, with all samples compared to the luciferase activity in the absence of any stimulation. The results represent the averages of three independent experiments, with the error bars reflecting the standard errors of the means. (B) Transfections were performed as in panel A, except that all samples were also stimulated with anti-TCR (prebound to the plate as described in Materials and Methods). (C) Transfections were performed as in panel A, except that all samples were also stimulated with 1 mM ionomycin. (D) Transfections were performed as in panel C, except that the murine T-cell lymphoma EL-4 was used and the anti-murine-CD28 monoclonal antibody 37.51 was used for stimulation.

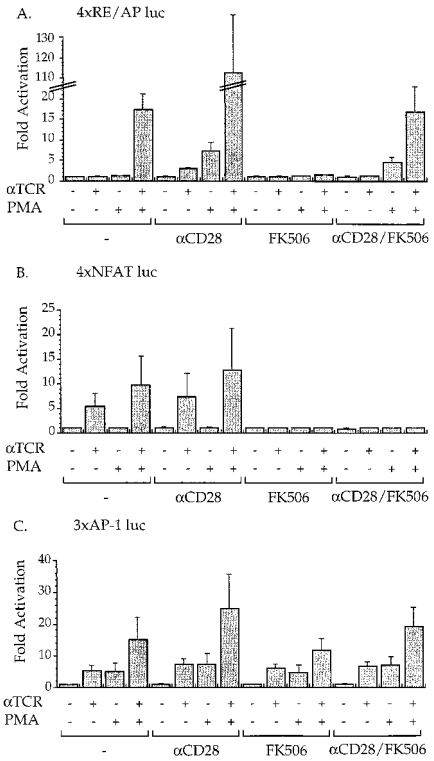

FIG. 3. FK506 affects RE/AP stimulation by anti-TCR (αTCR) and ionomycin but not by PMA or anti-CD28 (αCD28). (A) Transfections were performed as described in the legend for Fig. 2A with a 4xRE/AP luciferase (luc) reporter. Samples were left unstimulated (−) or were stimulated (+) with 5 ng of PMA or anti-TCR (prebound to the plate as described in Materials and Methods) per ml. Some samples were also stimulated with anti-CD28 (9.3) in the presence or absence of 100 ng of FK506, as indicated below the graph. (B) Transfections were performed as in panel A with a 4xNFAT luc reporter. (C) Transfections were performed as in panel A with a 3xAP-1 luc reporter.

specific monoclonal antibody 9.3. Thus, there appears to be an exact correlation between increased IL-2 production and transcriptional upregulation of RE/AP by various mutants of CD28, implying that the increase in IL-2 is mediated transcriptionally through the RE/AP composite element.

Since the RE/AP composite element mediates CD28 transcriptional activation of the IL-2 promoter, we were interested in identifying the proteins involved in this upregulation. Other studies have implicated the involvement of NFAT family members at the CD28RE (6, 24). However, our results (Fig. 3), in which the activation profiles of the composite RE/AP element and NFAT are quite distinct, argue against a role for NFAT proteins in RE/AP regulation. It has also been reported that NF-κB family members can activate transcription from the CD28RE (5, 17). To determine whether NF-κB family members can also activate transcription of the RE/AP composite element, the NF-κB family members p50, p65, and c-Rel were overexpressed in Jurkat cells and the effect on RE/AP reporter activity was examined. Both p65 and c-Rel overexpression had a dramatic effect on RE/AP activation, increasing it by approximately 3 orders of magnitude (Fig. 5). In contrast, p50 overexpression did not have an effect on RE/AP reporter activity. Surprisingly, activation by p65 or c-Rel was dependent only on the presence of a wild-type CD28RE; both p65 and c-Rel overexpression activated a mutant RE/AP reporter construction in which the AP-1 site alone was mutated (Fig. 5, construct RE/AP_m), while mutation of the CD28RE within the composite RE/AP element abolished the effect (constructs RE_m/AP and RE_mAP_m). These results differ from the response to anti-TCR and anti-CD28 stimuli where both the CD28RE and the adjacent AP-1 sites were absolutely required for transcriptional activity (Fig. 1B). Since overexpression of either p65 or c-Rel can stimulate activity from the mutated RE/AP_m reporter, which could not be otherwise stimulated by any combination of antibodies or pharmacological agents, the activation observed upon overexpression by either p65 or c-Rel does not reflect the events in vivo that occur upon T-cell activation. Therefore, one cannot conclude from these overexpression

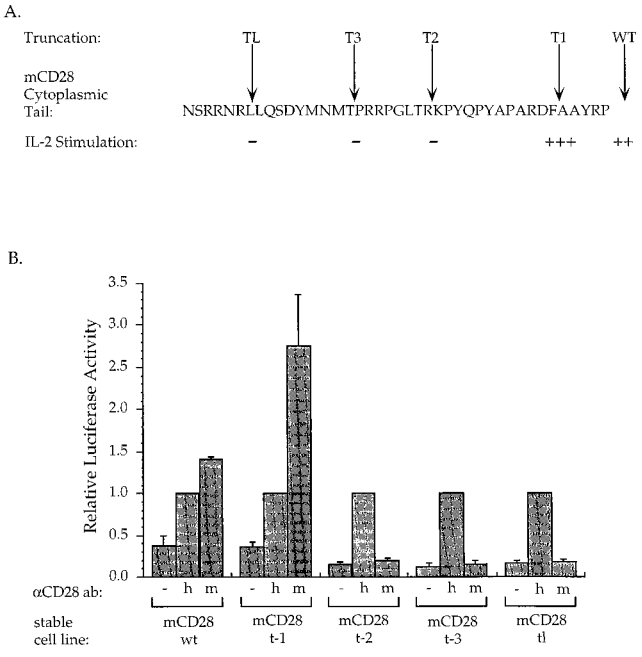

FIG. 4. Transcriptional activation of RE/AP by CD28 occurs only in those mutants that upregulate IL-2 secretion. (A) Shown schematically are the CD28 truncations used and their effects on IL-2 secretion (+, intact costimulatory function; −, no costimulatory function). These results are summarized from reference 31. MCD28, murine CD28. (B) Jurkat T cells (10^7) which stably expressed wild-type murine CD28 or various truncations (T1, T2, T3, or TL) were transfected with 20 μg of 4xRE/AP. Forty to 48 h later, 10^5 live cells per sample were stimulated with anti-TCR and 5 ng of PMA per ml. Additionally, some samples also received either anti-human or anti-murine CD28 antibodies (αCD28 ab; as indicated below the graph by an h or m, respectively). Cells were stimulated for 6 h. The data is shown as relative luciferase activity, with each value divided by the luciferase activity obtained when the cells were stimulated with PMA, anti-TCR, and anti-human CD28 (=1.0). The results represent the averages of three independent experiments, with the error bars representing the standard errors of the means.

studies whether p65 or c-Rel is physiologically relevant to RE/AP activation.

To determine which, if any, NF-κB family members are involved in transcriptional activation through the RE/AP com-

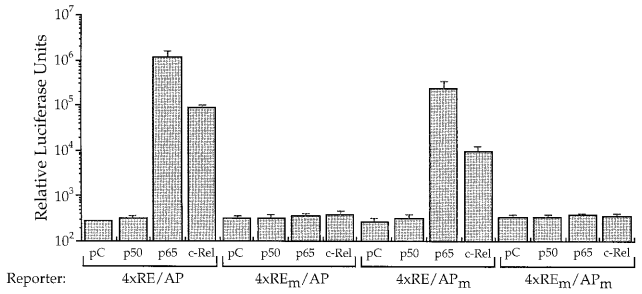

FIG. 5. p65 and c-Rel overexpression can activate transcription through the CD28RE alone. Jurkat T cells (10^7) were transfected with 20 μg of 4xRE/AP, 4xRE$_m$/AP, 4xRE/AP$_m$, or 4xRE$_m$/AP$_m$ luciferase reporter construct in combination with 5 μg of control vector pCDNA3, pCMV p50, pCMV4 p65, or pCMV4 c-Rel expression constructs. Forty to 48 h later, 10^5 live cells were examined for luciferase activity. Cells were stimulated for 6 h. The data is shown as relative luciferase activity, with all samples compared to the transfection with 4xRE/AP and pCDNA3 vector control (=1.0). The results represent the averages of three independent experiments, with the error bars representing the standard errors of the means.

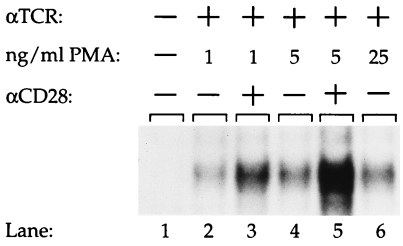

FIG. 6. Stimulation induces the binding of a complex to RE/AP. ^{32}P-radiolabelled RE/AP oligonucleotide was used as a probe in gel EMSAs. Five micrograms of nuclear extract was stimulated for 4 h with either anti-TCR (αTCR), anti-CD28 (αCD28), or PMA as indicated above the gel shift. Nonspecific noninduced bands and the free-probe band are not shown.

posite element, we sought to identify the transcription factors which bind to the CD28RE site in RE/AP in response to stimulation by using EMSAs. As shown in Fig. 6, an inducible complex could be observed binding to the RE/AP composite element when nuclear extracts from Jurkat T cells stimulated under conditions that were shown previously to activate transcription were used (Fig. 2). The additional stimulation by anti-CD28 greatly increased the intensity of this induced broad band (compare lane 3 to lane 2 and lane 5 to lane 4). No specific complex was detected in response to anti-CD28 stimulation. To further characterize the induced complex, oligonucleotide probes of the RE/AP composite element containing mutations at either the CD28RE or NF-IL-2B AP-1 site, or both, were used (Fig. 7). When the AP-1 site alone was mutated (RE/AP$_m$), a specific anti-CD28-induced band was revealed (Fig. 7; compare lane 9 with lane 8 or 7). Mutation of the CD28RE did not interfere with an induced complex binding to the AP-1 site of RE/AP, but this was induced by anti-TCR–PMA stimulation alone and was not further increased upon addition of anti-CD28. Mutation of both the CD28RE and the adjacent NF-IL-2B AP-1 sites completely abrogated all inducible binding of this complex. Hence, the one band induced by anti-TCR–PMA–anti-CD28 appears to actually be composed of two separate bands, which here only become distinguishable upon mutagenesis of RE/AP: an AP-1 complex bound to the NF-IL-2B site that is induced by anti-TCR–PMA and a complex bound to the CD28RE of almost identical mobility that is induced by anti-CD28.

To determine whether the proteins bound to the CD28RE belonged to the NF-κB family of transcription factors, EMSAs were performed with antibodies specific to p50, p65, or c-Rel. NRS was included as a negative control. Only the anti-c-Rel

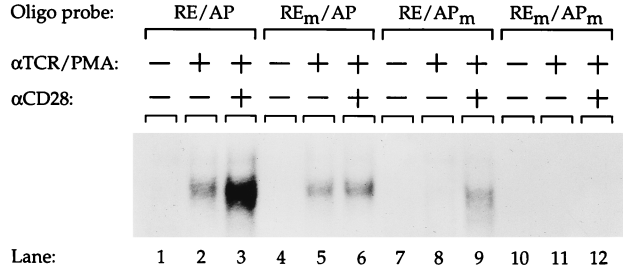

FIG. 7. A specific complex can be induced by anti-CD28 stimulation. ^{32}P-radiolabelled RE/AP, RE$_m$/AP, RE/AP$_m$, and RE$_m$/AP$_m$ oligonucleotides were used as probes in gel EMSAs. Five micrograms of nuclear extract was stimulated for 4 h with either anti-TCR (αTCR), anti-CD28 (αCD28) ascitic fluid, or 1 ng of PMA per ml (as indicated above the gel shift). Nonspecific noninduced bands and the free-probe band are not shown.

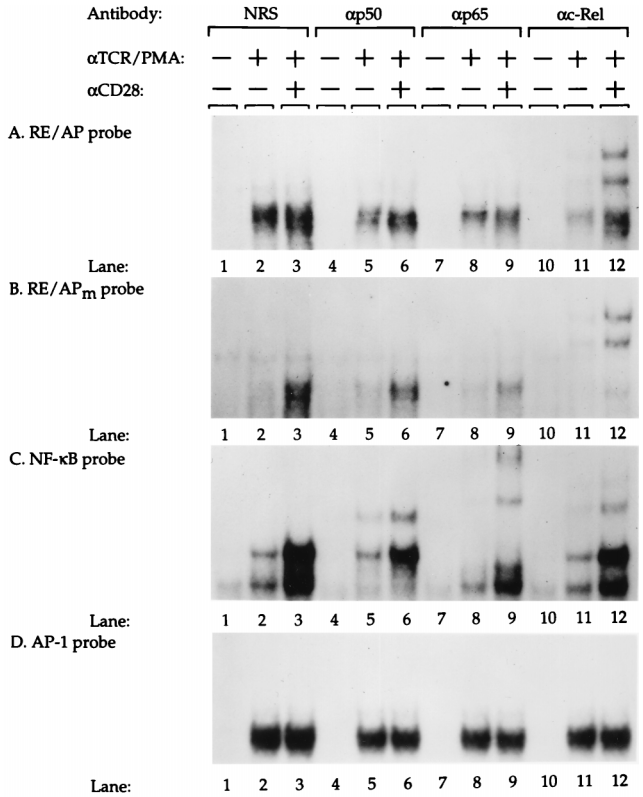

FIG. 8. c-Rel is the predominant protein binding to the CD28RE. (A) EMSAs were performed with an RE/AP probe as described in the legend for Fig. 6. In addition, all samples were incubated with either NRS, anti-p50 (αp50), anti-p65, or anti-c-Rel antibodies as indicated above the gel shift. (B) EMSAs were performed with the mutant RE/AP$_m$ probe as described in the legend for Fig. 6. In addition, all samples were incubated with either NRS, anti-p50, anti-p65, or anti-c-Rel antibodies as indicated above the gel shift. The slight decrease in the complex with the addition of anti-p65 antisera (lane 9) was not found consistently when this experiment was repeated. (C) EMSAs were performed with the consensus NF-κB site from the IL-2Rα promoter as a probe as described in the legend for Fig. 6. In addition, all samples were incubated with either NRS, anti-p50, anti-p65, or anti-c-Rel antibodies as indicated above the gel shift. (D) EMSAs were performed with the consensus AP-1 site from the metallothionein promoter as a probe as described in the legend for Fig. 6. In addition, all samples were incubated with either NRS, anti-p50, anti-p65, or anti-c-Rel antibodies as indicated above the gel shift.

antibodies supershifted proteins bound to RE/AP (Fig. 8A). These supershifted bands were primarily detected when the nuclear extracts were stimulated with anti-CD28 in addition to anti-TCR–PMA (lane 12). When nuclear extract stimulated with anti-TCR–PMA alone was used, only poor supershifting by anti-c-Rel antibodies was observed (lane 11). There was no detectable supershift with NRS, anti-p50, or anti-p65 antibodies. Therefore, c-Rel but not p50 or p65 appears to be in the induced complex bound to RE/AP. However, since the proteins bound to the AP-1 site within the RE/AP composite element comigrate with the complexes bound to the CD28RE within the RE/AP composite element, from these EMSAs, it is impossible to determine by the disappearance of the induced band whether c-Rel represents a majority or a minority of the induced complex at the CD28RE. Hence, the EMSAs were repeated with the mutated RE/AP$_m$ oligonucleotide as a probe. Similar to the results obtained with RE/AP as a probe, only the anti-c-Rel antibodies could supershift the complex induced by anti-TCR–PMA–anti-CD28 (Fig. 8B, lane 12). Interestingly, the anti-c-Rel antibodies shifted nearly all of the induced complex. Therefore, c-Rel is the major component of the induced complex binding to the CD28RE. To verify that the specific NF-κB antisera used could supershift the various family members, a control oligonucleotide corresponding to the NF-κB site from the IL-2Rα promoter was used in gel shift assays, in which binding of p50, p65, and c-Rel had been previously demonstrated (20). Supershifts induced by anti-p50, anti-p65, and anti-c-Rel were readily detected (Fig. 8C, lanes 6, 9, and 12). Additionally, similar to previously published work (5), stimulation through anti-CD28 greatly increased the NF-κB activity in the nuclear extracts (compare lane 3 with lane 2). Interestingly, the binding of all three NF-κB family members examined, p50, p65, and c-Rel, to the consensus NF-κB oligonucleotide was increased upon anti-CD28 stimulation. This is in direct contrast to what was found for the composite RE/AP element, where with anti-CD28 stimulation only the association of c-Rel was found. As a negative control, none of these antibodies had an effect on the binding of AP-1 proteins to a consensus AP-1 site (Fig. 8D). Therefore, while anti-CD28 stimulation had the general effect of increasing the binding of p50, p65, and c-Rel to a consensus NF-κB site, only c-Rel was found to bind specifically and inducibly to the nonconsensus NF-κB found within the RE/AP composite element.

DISCUSSION

We have demonstrated that CD28 responsiveness within the IL-2 promoter is conferred by a composite element composed of both the CD28RE and the adjacent NF-IL-2B AP-1 sites. Mutation of either the CD28RE or the NF-IL-2B AP-1 site abolished not only activation by CD28, but also stimulation by TCR, PMA, and/or ionomycin. Activation of RE/AP is not solely dependent upon CD28 stimulation; considerable activation also occurs by stimulation with the combination of PMA and either ionomycin or TCR. However, activation is maximally achieved when three signals are present: PMA, CD28, and either TCR or ionomycin. CD28-mediated activation of the RE/AP reporter is resistant to FK506 inhibition, consistent with CD28 costimulation of IL-2 production being FK506 insensitive. Therefore, RE/AP is a site of signal integration within the IL-2 promoter. The activation profile of the RE/AP reporter is separate and distinct from the activation profile of either an NFAT or AP-1 reporter, demonstrating that RE/AP represents a unique composite element. Using a panel of mutants of CD28, we also show an exact correlation between transcriptional upregulation of RE/AP and increases in IL-2 secretion induced by these mutants, implying a direct role for the RE/AP composite element in the transcriptional upregulation of the IL-2 promoter by CD28. We also examined which factors may bind and mediate transactivation of the CD28RE within RE/AP. Transient overexpression assays with the NF-κB family members p50, p65, and c-Rel demonstrate that both p65 and c-Rel but not p50 are potent activators of the RE/AP reporter construct. However, this activation is dependent only upon the presence of the wild-type CD28RE, not the AP-1 site, and therefore may not reflect events at the RE/AP composite element in vivo. Gel shift analysis, however, suggests that the major event induced by CD28 stimulation is c-Rel association with the CD28RE within the RE/AP composite element.

Interestingly, when either the CD28RE or the NF-IL-2B AP-1 site was mutated in RE/AP, the ability of the reporter to be activated was completely abolished (Fig. 1). Activation of a consensus AP-1 reporter alone (23, 30) by CD28 costimulation has been previously demonstrated. The NF-IL-2B AP-1 site, however, is a nonconsensus AP-1 site and therefore might not

function equivalently to a consensus AP-1 site. Consistent with this possibility, a multimerized NF-IL-2B site could not be activated to the same extent as a consensus AP-1 reporter (28a). Since mutation of the NF-IL-2B site in RE/AP eliminates both activation and CD28 upregulation, the presence of a wild-type NF-IL-2B site greatly affects stimulation through the CD28RE. Hence, CD28 transcriptional activation of the IL-2 promoter is mediated by a composite element containing both the CD28RE and the nonconsensus NF-IL-2B AP-1 sites. The functional requirement for both sites does not appear to involve cooperative binding of transcription factors to the CD28RE and NF-IL-2B sites, since mutation of one site does not eliminate binding of factors to the other site (Fig. 7). However, we cannot eliminate the possibility that cooperative binding does occur when multimerized sites are used, as in our transcriptional activation assays.

The identification of c-Rel by gel shift assays as the major factor binding to the CD28RE is in apparent conflict with the previously published work demonstrating that both p50 and p65 can associate with the CD28RE (5, 17). Since the CD28RE is an NF-κB-like site, it is not surprising that under different conditions such as UV cross-linking (5), in vitro interactions with many NF-κB family members can potentially occur. However, our results demonstrate that under conditions where CD28 upregulates the nuclear expression of p50, p65, and c-Rel (as shown by increased binding to the NF-κB site from the IL-2Rα promoter), only c-Rel is associated with the CD28RE in gel shift assays. Therefore, the CD28RE appears to have a preference for c-Rel over other NF-κB family members. It has also been shown that the CD28RE may represent a novel site for NFAT binding (24). However, the differences in the activation requirements between the RE/AP and NFAT reporters argue against a functional role for NFAT in activation through the CD28RE (Fig. 3). Again, since the NFAT DNA binding domain is distantly related to the rel homology domain, it is not surprising that under certain in vitro conditions an association of NFAT protein with the CD28RE can occur. Differences between our results and those of others (6, 24) may also reflect the differences in the cell types and stimulations used. Our data demonstrating a preference of the CD28RE for c-Rel agrees with recent work showing that the high-mobility group protein I(Y) preferentially enhances the binding of c-Rel to the CD28RE within the IL-2 and granulocyte-macrophage colony-stimulating factor promoters (8). Finally, our results suggesting a role for c-Rel in the function of RE/AP are consistent with the failure of c-Rel-deficient T cells to produce IL-2 (16), a phenotype not observed with either p50 or NFATp-deficient T cells (9, 26, 27, 34).

Arguments have also been made for the involvement of p65, c-Rel, and NFAT in the activation of the CD28RE on the basis of transient overexpression experiments (5, 6, 17). Consistent with this, we found that overexpression of either p65 or c-Rel had a profound effect on activation of RE/AP (Fig. 5). However, activation occurred even when the NF-IL-2B AP-1 site was mutated (RE/AP_m) in these overexpression experiments. Moreover, in our studies in which PMA, anti-TCR, and anti-CD28 stimuli were used, only binding of c-Rel and not p65 to the CD28RE was detected, even though CD28 upregulated the nuclear binding of both p65 and c-Rel to a different NF-κB site (Fig. 8). Therefore, we believe that c-Rel and not p65 is the physiologically relevant NF-κB family member associating with CD28RE. Most overexpression experiments measure activation 1 to 2 days after transfection, sufficient time for overexpression to set off a cascade of secondary events which then lead to reporter activation. Therefore, overexpression alone is not the definitive proof of direct involvement of a particular transcription factor with an enhancer element. Within our own experiments, overexpression of p65 or c-Rel led to activation of the mutated RE/AP_m reporter. However, this mutated RE/AP_m was not transcriptionally activated under any stimulation conditions examined. Therefore, activation solely dependent upon c-Rel or p65 is not likely to occur in vivo.

While our results clearly demonstrate a transcriptional upregulation by CD28 of the composite RE/AP element from the IL-2 promoter, others have not been able to demonstrate any activation of transcription by CD28 (32). This may represent differences unique to the system in which the experiments were performed; transcriptional upregulation of the IL-2 promoter by CD28 has been demonstrated in human Jurkat T-cell clones (2, 3) and in human peripheral blood T cells (3, 10), while the lack of a transcriptional effect has been obtained primarily from studies utilizing long-term murine T-cell clones (15, 32). However, transcriptional activation by CD28 has been demonstrated in murine T cells from a transgenic mouse containing an AP-1 luciferase reporter (23). Additionally, we also observed CD28-mediated upregulation of the RE/AP composite element in the murine T-cell line EL-4 (Fig. 2D). Clarification of the role of CD28 and c-Rel in transcriptional upregulation of the IL-2 promoter through the RE/AP composite element in primary murine cells will require the generation of an RE/AP reporter transgenic mouse, which is in progress.

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REFERENCES

1. **Civil, A., A. Bakker, I. Rensink, A. Doerre, L. A. Aarden, and C. L. Verweij.** 1996. Nuclear appearance of a factor that binds the CD28 response element within the interleukin-2 enhancer correlates with interleukin-2 production. J. Biol. Chem. **271:**8321–8327.
2. **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 signals. Eur. J. Immunol. **22:**3041–3043.
3. **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.
4. **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.
5. **Ghosh, P., T.-H. Tan, N. R. Rice, A. Sica, and H. A. Young.** 1993. The interleukin 2 CD28-responsive complex contains at least three members of the NF-κB family: c-Rel, p50 and p65. Proc. Natl. Acad. Sci. USA **90:**1696–1700.
6. **Good, L., S. B. Maggirwar, and S.-C. Sun.** 1996. Activation of the IL-2 gene promoter by HTLV-I Tax involves induction of NF-AT complexes bound to the CD28-responsive element. EMBO J. **15:**3744–3750.
7. **Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, 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.
8. **Himes, S. R., L. S. Coles, R. Reeves, and M. F. Shannon.** 1996. High mobility group protein I(Y) is required for function and for c-Rel binding to CD28 response elements within the GM-CSF and IL-2 promoters. Immunity **5:**479–489.
9. **Hodge, M. R., A. M. Ranger, F. C. de la Brousse, T. Hoey, M. J. Grusby, and L. H. Glimcher.** 1996. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. Immunity **4:**397–405.
10. **Hughes, C. C. W., and J. S. Pober.** 1996. Transcriptional regulation of the interleukin-2 gene in normal human peripheral blood T cells. J. Biol. Chem. **271:**5369–5377.
11. **Jain, J., C. Loh, and A. Rao.** 1995. Transcriptional regulation of the IL-2

gene. Curr. Opin. Immunol. **7:**333–342.
12. **Jain, J., V. E. Valge-Archer, and A. Rao.** 1992. Analysis of the AP-1 sites in the IL-2 promoter. J. Immunol. **148:**1240–1250.
13. **Jenkins, M. K.** 1994. The ups and downs of T cell costimulation. Immunity **1:**443–446.
14. **June, C. H., J. A. Bluestone, L. M. Nadler, and C. B. Thompson.** 1994. The B7 and CD28 receptor families. Immunol. Today **15:**321–331.
15. **Kang, S.-M., B. Beverly, A.-C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo.** 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science **257:**1134–1138.
16. **Kontgen, F., R. J. Grumont, A. Strasser, D. Metcalf, R. Li, D. Tarlinton, and S. Gerondakis.** 1995. Mice lacking the *c-rel* proto-oncogene exhibit defects in lymphocyte proliferation, humoral immunity, and interleukin-2 expression. Genes Dev. **9:**1965–1977.
17. **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.
18. **Lasalle, J. M., and D. A. Hafler.** 1994. T cell anergy. FASEB J. **8:**601–608.
19. **Meyer, C. M., X. Wang, C. Chang, D. Templeton, and T.-H. Tan.** 1996. Interaction between c-Rel and the mitogen-activated protein kinase kinase kinase 1 signaling cascade in mediating κB enhancer activation. J. Biol. Chem. **271:**8971–8976.
20. **Pierce, J. W., C. A. Jamieson, J. L. Ross, and R. Sen.** 1995. Activation of IL-2 receptor α-chain gene by individual members of the *rel* oncogene family in association with serum response factor. J. Immunol. **155:**1972–1980.
21. **Rao, A.** 1994. NFATp: a transcription factor required for the coordinate induction of several cytokine genes. Immunol. Today **15:**274–281.
22. **Riegel, J. S., B. Corthesy, W. M. Flanagan, and G. R. Crabtree.** 1992. Regulation of the interleukin-2 gene. Chem. Immunol. **51:**266–298.
23. **Rincon, 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.
24. **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.
25. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schattner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
26. **Serfling, E., A. Avots, and M. Neumann.** 1995. The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. Biochim. Biophys. Acta **1263:**181–200.
27. **Sha, W. C., H.-C. Liou, E. I. Tuomanen, and D. Baltimore.** 1995. Targeted disruption of the p50 subunit of NF-κB leads to multifocal defects in immune responses. Cell **80:**321–330.
28. **Shapiro, V. S., M. N. Mollenauer, W. C. Greene, and A. Weiss.** 1996. c-Rel regulation of IL-2 gene expression may be mediated through activation of AP-1. J. Exp. Med. **184:**1663–1670.

28a. **Shapiro, V. S., and A. Weiss.** Unpublished results.

29. **Springer, T. A.** 1990. Adhesion receptors of the immune system. Nature **346:**425–434.
30. **Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah.** 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell **77:**727–736.
31. **Truitt, K. E., T. Nagel, L. F. Suen, and J. B. Imboden.** 1996. Structural requirements for CD28-mediated costimulation of IL-2 production in Jurkat T cells. J. Immunol. **156:**4539–4541.
32. **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:**177–197.
33. **Weiss, A., and D. R. Littman.** 1994. Signal transduction by lymphocyte antigen receptors. Cell **76:**263–274.
34. **Xanthoudakis, S., J. P. B. Viola, K. T. Y. Shaw, C. Luo, J. D. Wallace, P. T. Bozza, T. Curran, and A. Rao.** 1996. An enhanced immune response in mice lacking the transcription factor NFAT1. Science **272:**892–895.