

The AP-1 Site at -150 bp, but Not the NF- κ B Site, Is Likely to Represent the Major Target of Protein Kinase C in the Interleukin 2 Promoter

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Summary

Stimulation of T cells with antigen results in activation of several kinases, including protein kinase C (PKC), that may mediate the later induction of activation-related genes. We have examined the potential role of PKC in induction of the interleukin 2 (IL-2) gene in T cells stimulated through the T cell receptor/CD3 complex. We have previously shown that prolonged treatment of the untransformed T cell clone Ar-5 with phorbol esters results in downmodulation of the α and β isozymes of PKC, and abrogates induction of IL-2 mRNA and protein. Here we show that phorbol ester treatment also abolishes induction of chloramphenicol acetyltransferase activity in Ar-5 cells transfected with a plasmid containing the IL-2 promoter linked to this reporter gene. The IL-2 promoter contains binding sites for nuclear factors including NFAT-1, Oct, NF- κ B, and AP-1, which are all potentially sensitive to activation of PKC. We show that induction of a trimer of the NFAT and Oct sites is not sensitive to phorbol ester treatment, and that mutations in the NF- κ B site have no effect on inducibility of the IL-2 promoter. In contrast, mutations in the AP-1 site located at -150 bp almost completely abrogate induction of the IL-2 promoter, and appearance of an inducible nuclear factor binding to this site is sensitive to PKC depletion. Moreover, cotransfections with *c-fos* and *c-jun* expression plasmids markedly enhance induction of the IL-2 promoter in minimally stimulated T cells. Our results indicate that the AP-1 site at -150 bp represents a major, if not the only, site of PKC responsiveness in the IL-2 promoter.

Stimulation of T lymphocytes by antigen results in the activation of serine/threonine and tyrosine kinases, and later in induction of a large number of activation-associated genes (reviewed in references 1-3). One of the best characterized of the kinases is protein kinase C (PKC),¹ a family of phospholipid-dependent serine/threonine kinases that are activated as a consequence of increased phosphatidylinositol turnover in stimulated T cells (reviewed in references 4 and 5). Gene induction in activated T cells is at least partly mediated by PKC, since phorbol esters (alone or in combination with calcium ionophores) can activate PKC and gene expression in parallel (reviewed in references 3 and 6). In fact, phorbol esters induce a variety of transcription factors that are capable of binding to specific DNA sequences in the promoter regions of phorbol ester-inducible genes. Among these is the AP-1 (Fos/Jun) transcription factor, which is newly synthesized as well as posttranslationally modified in phorbol ester-stimu-

lated cells (7, 8), and the NF- κ B transcription factor, which is released from a preexisting cytosolic complex with an inhibitory protein upon phorbol ester treatment (9).

The gene for the lymphokine IL-2, a growth factor for T and B cells, is induced within 1-6 h after TCR stimulation (10, 11), and represents a good model system for investigating the relation between PKC activation and lymphokine gene induction in T cells. The sites relevant for induction of the IL-2 promoter have been exhaustively mapped. Both constitutive (Oct) and inducible (NFAT-1, NF- κ B, AP-1, and CD28RC) DNA binding factors have been implicated in induction (11-19). Each of these inducible factors can be induced by phorbol esters and/or calcium ionophores in T cells and other cell types, suggesting the potential involvement of PKC and/or calcium-dependent kinases. Moreover the induction of *c-Fos*, a component of the AP-1 family of transcription factors (7), is PKC dependent in T cells (20).

By itself, the use of phorbol esters has not been sufficient to prove the involvement of PKC, since the activity of several other kinases and enzymatic processes can be secondarily regu-

¹ Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; PKC, protein kinase C; hGH, human growth hormone.

lated, either positively or negatively, by PKC (reviewed in references 2, 5, and 21). In fact the changes in phosphorylation of c-Jun in phorbol ester-stimulated fibroblasts cannot be replicated *in vitro* with purified PKC (8). Experiments with PKC inhibitors have also been equivocal, since few inhibitors are entirely selective for PKC, and most are toxic to untransformed cells at concentrations approaching those required for complete inhibition of kinase activity (22). An alternative method for assessing the involvement of PKC has relied on downmodulation of certain PKC isozymes by prolonged treatment with high concentrations of phorbol esters (23), a technique that works particularly well in untransformed cells with low biosynthetic rates. The mechanism of downmodulation is apparently through recruitment of essentially all the enzyme to the plasma membrane, where it is rapidly degraded by a protease (24, 25). Although untransformed cells treated in this manner show an initial acute response to the phorbol ester, this response fades rapidly. After 36–48 h, the treated cells appear similar to quiescent, untreated T cells in their growth rates, basal levels of cellular kinase activity, and expression of activation-associated genes (20, 26).

In murine thymocytes, Western blotting with isozyme-specific antibodies indicates that the α and β PKC isozymes are downmodulated by chronic phorbol ester treatment, and expression of the ϵ isozyme is unaffected (27). Depletion of the α and β isozymes by phorbol ester treatment has been confirmed by direct measurements of PKC activity in untransformed murine T cell clones (20, 26). The residual ϵ isozyme was not detected since it does not efficiently phosphorylate the histone substrate used in the standard assays for PKC (28). T cells chronically treated with phorbol esters, and thus depleted for the α and β PKC isozymes, show no gene induction or lymphokine secretion in response to TCR ligands (20, 26), suggesting that these downmodulated isozymes are required for signal transduction through the TCR. A less likely explanation is that chronic phorbol ester treatment affects an unrelated process that is independently required for gene induction and lymphokine secretion in activated T cells.

We have used the untransformed, antigen-responsive T cell clone Ar-5 (20, 29, 30) to identify the PKC-sensitive sites in the IL-2 gene promoter. Ar-5 cells show low constitutive but strong inducible expression of nuclear factors that bind to sites in the IL-2 promoter. Moreover, induction of chloramphenicol acetyltransferase (CAT) activity in Ar-5 cells transfected with an IL-2 promoter-CAT plasmid, and then activated through the TCR, is completely suppressed by chronic phorbol ester treatment, thus implicating the α and β isozymes of PKC in IL-2 gene induction and facilitating the identification of potentially PKC-sensitive mechanisms for IL-2 gene induction. We show that a major locus of PKC responsiveness in the IL-2 promoter is likely to be the AP-1 site located at –150 bp upstream of the transcription start site. Analysis of promoter mutations indicates that an intact NF- κ B site is not required for IL-2 gene induction in Ar-5 T cells.

Materials and Methods

Plasmids. The human 5' IL-2-CAT plasmid (obtained from G. Crabtree, Stanford University, Stanford, CA) contains 635 bp of the human IL-2 promoter linked to the CAT gene and SV40 poly(A) tail as described (31). The murine 5' IL-2-CAT plasmids pIL-CAT0, pIL-CAT2/1⁺, and pIL-CAT2/1-M (obtained from E. Serfling, Universität Würzburg, Würzburg, Germany) contain –2000 to –7 bp, –293 to –7 bp, and –293 to –200 bp region, respectively, of the murine IL-2 promoter linked to the Herpes simplex virus thymidine kinase (tk) promoter placed upstream of the CAT gene in the plasmid pBLCAT2 (14). The three human 5' IL-2-CAT plasmids bearing mutations in the IL-2 κ B site (obtained from M. Siekevitz, Mount Sinai Medical Center, New York, NY) were as described previously (13). The four mutants in the –150 and –180 bp AP-1 sites were constructed in the human 5' IL-2-CAT plasmid (30, 31) using uracil-labeled template DNA, as described (32). Mutated plasmids were selected by colony hybridizations using the mutagenic oligonucleotide, and their identity was confirmed by restriction mapping and sequencing (33). The oligonucleotides used for the site-directed mutagenesis of the AP-1 sites were: M1: 5'CAAAGACTGACTTTATGGTA, M2: 5'CAAAGGGTGACTGAATGGAT, M3: 5'TCTGATGACTCGAGGGAATT, and M4: 5'TCTGATGTTGCTTTGGAATT. The expression plasmids BK28 (human c-Fos, [34]) and pRSV-cJ (human c-Jun, [35]) were used for cotransfections. The control plasmids that lacked the c-Fos and c-Jun coding sequences, but possessed the FBJ-MSV proviral LTR and the RSV LTR, respectively, were kindly provided by Dr. E. Flemington, Dana-Farber Cancer Institute, Boston, MA.

Transfections. The murine T cell clone Ar-5 was cultured in "T cell medium," consisting of DMEM containing 10% FCS and rat T cell growth factor (CR-rTCGF, Collaborative Research Inc., Lexington, MA) as a source of IL-2, and other additives, as described previously (29, 30, 36). For PKC depletion, cells were treated with 200 nM PMA for 2 d before transfection, and 200 nM PMA was included in the culture medium until the cells were harvested. For transfections, untreated or PMA-treated cells ($2-3 \times 10^7$) were harvested in log phase growth ($\sim 5 \times 10^5$ cells/ml), incubated with 10–15 μ g of DNA and 100 μ g/ml DEAE-dextran in 2 ml DMEM for 30 min at 37°C, and split equally into two flasks containing 10 ml of T cell medium and coated with rabbit anti-hamster antibodies, if appropriate. The transfected cells were activated the next day, harvested 24 h later, and assayed for CAT activity, as described (37). The conversion of ¹⁴C-chloramphenicol to its acetylated forms was quantified using a Betagen Betascope (Waltham, MA). To control for the increase in protein synthesis in activated cells, the protein concentration of cell extracts was measured according to Bradford (38) and used to normalize the measured CAT activity. In addition, the plasmid pRSVCAT (37) was used as a standard for transfection efficiency of the cells in each experiment (30). The enhancerless plasmid pBL-CAT2 (39) was not inducible in transfections and exhibited very low basal transcriptional activity.

The stimuli used for activation were antigen (arsonate-conjugated OVA, 100 μ g/ml) with 2×10^6 TA3 APC (29, 36), anti-CD3 ϵ (1% 145-2C11 hybridoma supernatant [40]) crosslinked either via Fc receptors on 2×10^6 TA3 cells or on flasks coated with 300 μ g/ml rabbit anti-hamster antibody (Cappel Laboratories, Cochranville, PA), or 10-nM PMA plus 100 nM ionomycin. Activation was monitored in all experiments by measuring proliferation or IL-2 secretion (20, 36). We have noticed that transfected Ar-5 cells stimulated with TA3 cells and antigen or anti-CD3 ϵ show consistently lower induction of IL-2-CAT activity than cells stimulated with

anti-CD3 ϵ that have been crosslinked using immobilized second antibody, possibly because the latter stimulus is capable of cross-linking a larger proportion of surface TCR/CD3 complexes. Nevertheless, all three stimuli yield qualitatively very similar results. For most experiments, the use of anti-CD3 crosslinked with second antibody was preferred, because it monitors activation solely through the TCR/CD3 complex, uncomplicated by interactions involving other cell-surface receptors such as CD28 (19).

The quality of the plasmid DNAs used for transfections was monitored by checking them all for equivalent content of supercoiled DNA by agarose gel electrophoresis, and by cotransfecting them with an RSV-hGH (human growth hormone) internal control plasmid and monitoring hGH activity in supernatants and extracts of unstimulated cells as described (41). Values for intracellular growth hormone ranged from 3 to 11 ng/2 \times 10⁷ cells 48 h after transfection, and values of secreted hGH ranged from 1.85 to 5.1 ng/2 \times 10⁷ cells. At least three independent DNA preparations were tested for each plasmid. Relative to the wild-type human 5' IL-2-CAT plasmid (set to 100%), the transfection efficiency of the IL-2 κ B mutants was as follows: M1 (80.1%), M4 (62.1%), and M5 (60.9%). Similarly, relative to the wild-type human 5' IL-2-CAT plasmid (set to 100%), the transfection efficiency of the AP-1 mutants monitored by this assay was as follows: M1 (92.6%), M2 (131.4%), M3 (265.4%), and M4 (123.4%). Cotransfection with internal control plasmids could not be performed in our experiments, since all the internal control plasmids that we have tested were induced in activated T cells, and since induction of the CAT plasmids was decreased when internal control plasmids were cotransfected, apparently because of competition between the CAT plasmids and the internal control plasmids for relevant transcription factors. These problems are likely to reflect lower resting, as well as lower inducible levels of transcription factors in untransformed T cell clones, compared with the transformed T cells previously used for studies of IL-2 promoter activation (JURKAT, EL-4, and LBRM-33 [11, 12, 14, 16]).

Electrophoretic Mobility Shift Assays. The following oligonucleotides were used as probes in gel-shift assays: Murine Ig κ enhancer κ B site (42): 5'gacCAGAGGGGACTTTCCGAGA; 3'Ig κ B mu-

tant: 5'gacCAGAGGGGACTTGAAGAGA; 5'Ig κ B mutant: 5'gacCAGAATTCACCTTCCGAGA; Wild-type murine IL-2 κ B site (TCE_d, [14]): 5'gacACCAAGAGGGATTTCACCTAAATCC; -150 bp murine AP-1 site (TRE_p, [14]): 5'gacAATTCCAGAGAGTCATCAGA; -180 bp murine AP-1 site (TRE_d, [14]): 5'gacTAAATCCATTCAGTCAGTGTATG; Murine NFAT-1 site (Pu-box_d, [14, 15]): 5'gacGCCCAAAGAGGAAAATTTGTTTCATACAG; Human metallothionein AP-1 site (43): 5'tcgaCGTGAC-TCAGCGCGC.

The oligonucleotides were endlabeled with γ [³²P]-ATP and polynucleotide kinase (33) and purified on 12% acrylamide gels. Small-scale nuclear extracts were made from 2 \times 10⁷ unactivated or activated Ar-5 cells by a modification of the method of Dignam (44). Unless otherwise stated, cells were activated for 2 h with anti-CD3 ϵ antibody which was crosslinked using immobilized second antibody. For each binding reaction, 10,000 cpm (\sim 0.2–0.5 ng) of endlabeled probe was incubated with 5–8- μ g nuclear extract in the presence of 3 μ g sheared poly(dI-dC). The binding reaction was performed for 5–10 min (AP-1), or 15–20 min (NFAT-1 and IL-2 κ B) at room temperature. The resulting complexes were analyzed by electrophoresis at room temperature on 4% acrylamide gels (33).

Results and Discussion

Induction of the IL-2 Promoter Is Sensitive to Chronic Phorbol Ester Treatment. The antigen-responsive murine T cell clone Ar-5 (29) was left untreated or depleted of PKC by prolonged treatment with a high concentration of PMA (200 nM for 48 h). As mentioned previously, this treatment results in selective degradation of the α and β isozymes of PKC, but not the ϵ isozyme in murine thymocytes (27), and abrogates induction of IL-2 mRNA in Ar-5 cells (20). Untreated and PMA-treated cells were transfected with plasmids containing the 5' sequences of either the human or murine IL-2 promoters linked to the CAT reporter gene (14, 31). The transfected

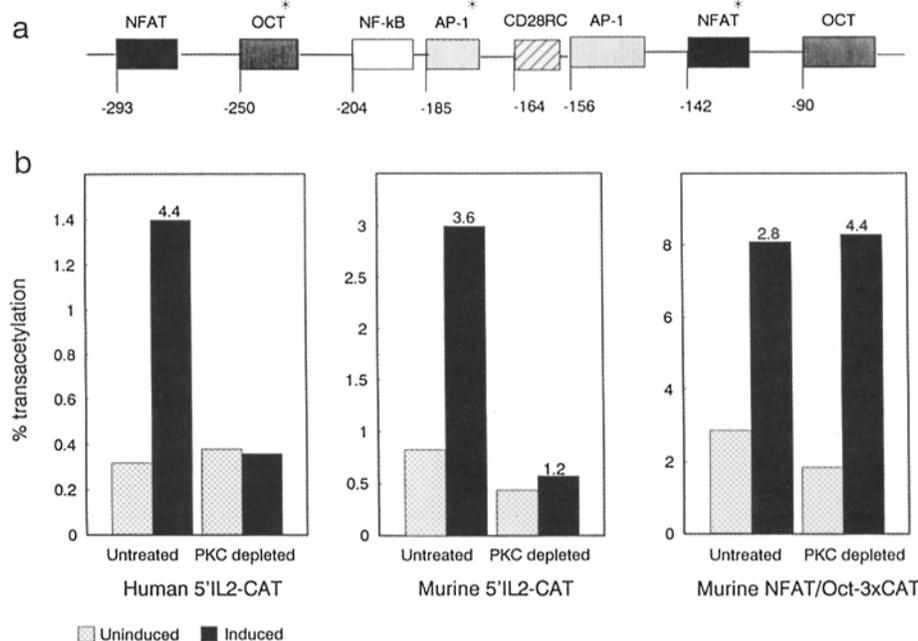
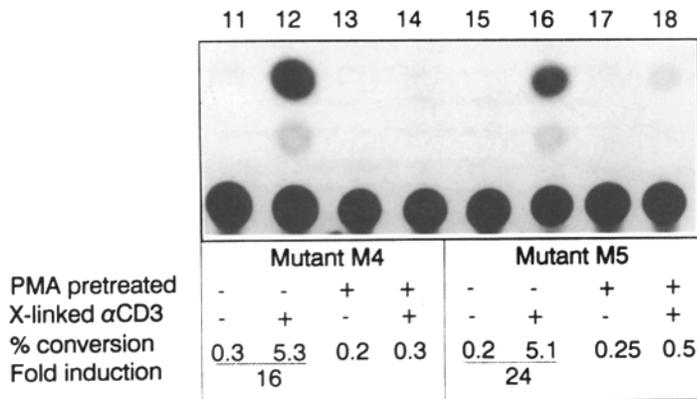
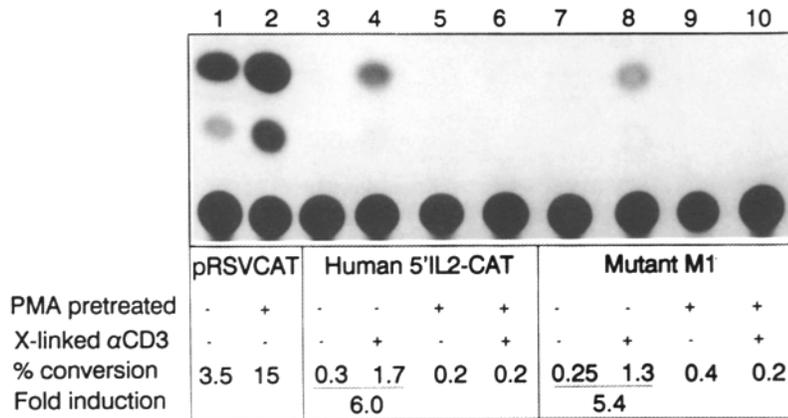
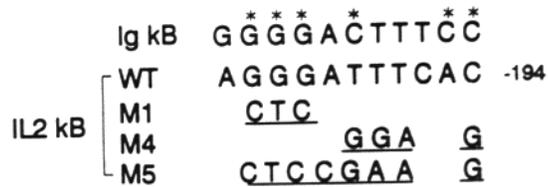


Figure 1. Sensitivity of the IL-2 promoter to PKC depletion by chronic phorbol ester treatment. (a) A schematic diagram of the IL-2 promoter, showing the locations of sites thought to be important for promoter induction. (*) Weaker binding sites. (b) Induction of CAT activity after transfection of the indicated plasmids into Ar-5 cells. Untreated cells or cells treated for 48 h with the phorbol ester PMA were transfected with human (left) or murine (center) 5'IL-2-CAT plasmids or with a plasmid containing a trimer of the NFAT and OCT sites (right), and stimulated with TA3 cells and antigen or anti-CD3 ϵ . A representative experiment using TA3 cells and anti-CD3 ϵ is shown. At least three similar experiments were performed with independent preparations of each plasmid.

a



cells were stimulated 24-h later with either antigen (arsonate-conjugated OVA; Ars-OVA) (29) presented on TA3 cells, or with a mAb to the TCR/CD3 complex (145-2C11, anti-CD3 ϵ) that was crosslinked using either TA3 cells or an immobilized second antibody. The cell extracts were assayed for CAT activity 18–24 h later (37).

Both human and murine 5' IL-2-CAT constructs were induced three–fivefold after activation of Ar-5 cells by TA3 cells and antigen or anti-CD3 (Fig. 1 *b*, left and center). No induction was seen if the cells were depleted of PKC by phorbol ester treatment (Fig. 1 *b*, left and center). This was expected since IL-2 mRNA and protein are not induced after chronic phorbol ester treatment (20). The plasmid pRSVCAT, which is constitutively expressed, was used as a control for transfection efficiency and was equivalently expressed in untreated and PMA-pretreated cells (see Fig. 2). The PMA-pretreated cells did not proliferate in response to PMA and ionomycin nor to anti-CD3 ϵ antibody, confirming that they were depleted of the relevant (α and β) PKC isozymes (data not shown).

Induction of an NFAT/Oct Trimer Is Insensitive to Chronic Phorbol Ester Treatment. The human and mouse IL-2 promoters share binding sites for several known transcription factors in the region extending \sim 300 bases upstream of the transcription start site (see Fig. 1 *a*). This region contains sites for the nuclear factors NFAT-1, Oct-1/Oct-2, NF- κ B, CD28RC, and AP-1 (11–19). NFAT-1, AP-1, and NF- κ B binding activities have been shown to be induced by phorbol esters and/or calcium ionophores in T cells (JURKAT [12, 13] and EL-4 cells [14]), and in other cell types (7, 45, 46), implicating PKC and/or calcium dependent kinases in their activation.

To determine whether the NFAT-1 and Oct sites contributed to the PKC responsiveness of the IL-2 promoter, we transfected a CAT plasmid (pIL-CAT2/1-M; [14]) containing a trimer of the murine NFAT-1/Oct site (-293 to -200 bp relative to the transcription start site) into untreated and phorbol ester-treated Ar-5 cells (Fig. 1 *b*, right). The NFAT-1/Oct construct was induced strongly under both conditions

b

Labelled probe : IL2 κ B A G G G A T T T C A C
 Competitors : Ig κ B G G G G A C T T T C C
 5' Mut A T T C
 3' Mut G A A

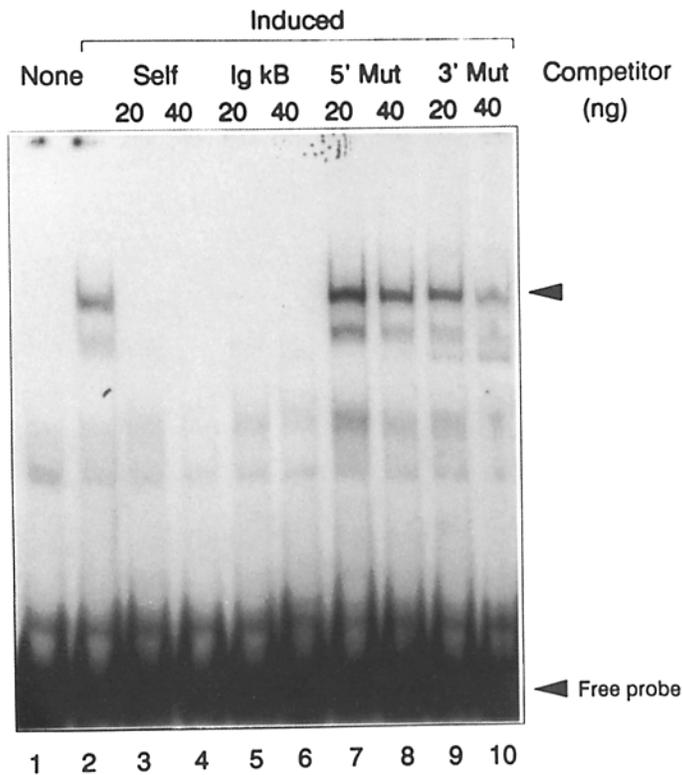


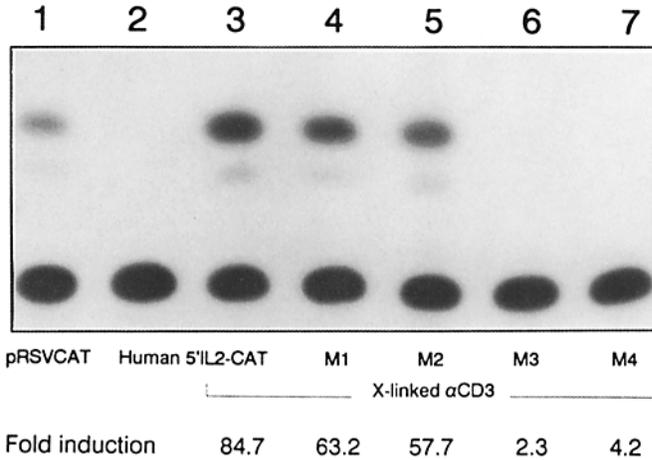
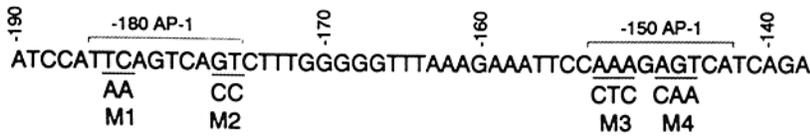
Figure 2. Induction of the IL-2 promoter is unaffected by mutations of the κ B site that abolish NF- κ B binding. (a) Induction of CAT activity from wild-type (lanes 3–6) and κ B site mutant promoters (lanes 7–18) after transfection into untreated Ar-5 cells (lanes 1, 3, 4, 7, 8, 11, 12, 15, and 16) or cells depleted of PKC by pretreatment with the phorbol ester PMA (lanes 2, 5, 6, 9, 10, 13, 14, 17, and 18). Cells were either unstimulated (lanes 1–3, 5, 7, 9, 11, 13, 15, and 17) or stimulated with anti-CD3 ϵ cross-linked with immobilized second antibody (lanes 4, 6, 8, 10, 12, 14, 16, and 18). One representative experiment of four performed with each plasmid, is shown. The plasmid pRSVCAT was used as a control for transfectability of the untreated (lane 1) or PMA downregulated Ar-5 cells (lane 2). (Top) Sequences of the κ B site mutants M1, M4, and M5 are compared with the sequences of the wild-type IL-2 κ B site and the Ig κ gene enhancer κ B site. (b) Binding of an inducible NF- κ B-like nuclear factor from Ar-5 cells to the IL-2 κ B site. Cells were untreated (lane 1) or activated with crosslinked anti-CD3 ϵ (lanes 2–10), nuclear extracts were prepared, incubated with a labeled oligonucleotide containing the IL-2 κ B site in the presence or absence of unlabeled competitors as indicated, and analyzed by electrophoretic mobility shift assay. (◀) Positions of the free probe and the inducible retarded complex. (Top) Sequences of the relevant regions of the IL-2 κ B probe and of the unlabeled competitors.

(Fig. 1, *b*, right). Although this experiment does not distinguish between the individual contributions of NFAT-1 and Oct sites, it is clear that downmodulation of PKC activity (or any other effect of chronic PMA pretreatment) does not compromise their enhancer function in Ar-5 cells. Likewise, phorbol ester treatment did not affect the induction of a protein complex that bound specifically to the NFAT-1 site in gel-shift assays (see Fig. 3 *b*, lanes 9–12). The lack of effect of PKC depletion on induction of the NFAT-1 site trimer, and on appearance of NFAT-1 binding activity in a gel-shift assay, is consistent with the suggestion that calcium-dependent processes may be involved in the activation of NFAT-1 (18). However PKC isozymes (such as the ϵ isozyme) that are not downmodulated by chronic phorbol ester treatment might also be involved (27).

Mutations in the NF- κ B Site Do Not Decrease IL-2 Promoter Induction. These experiments suggested that the NFAT and Oct sites were not PKC responsive (as defined by their insensitivity to chronic phorbol ester treatment), and hence that

the PKC-responsive site(s) were located downstream of –200 bp in the IL-2 promoter. Two likely candidates were the NF- κ B and AP-1 sites (Fig. 1 *a*), which can bind purified NF- κ B and AP-1 proteins as well as PMA-inducible nuclear factors from activated T cells in vitro (13, 14, 42, 47). We used site-specific mutants to determine the importance of the NF- κ B site in IL-2 promoter induction. Three 5' IL-2-CAT plasmids derived from mutations in the NF- κ B site of the human IL-2 gene (13) (Fig. 2 *a*, top) were transfected into Ar-5 cells. All three mutant promoters (Fig. 2 *a*, lanes 7, 8; 11, 12; and 15 and 16) could be activated at least as well as the wild-type promoter (lanes 3 and 4). The lack of effect of mutations in the site was not due to any differences in the ability of the plasmids to be transfected as determined by the internal controls (see Materials and Methods). In fact, mutants M4 and M5 (lanes 11, 12; and 15, 16) consistently showed two to fourfold higher induction than the wild-type promoter (lanes 3 and 4), despite being lower in transfection efficiency. This suggests that perhaps the IL-2 NF- κ B site is a target

a



b

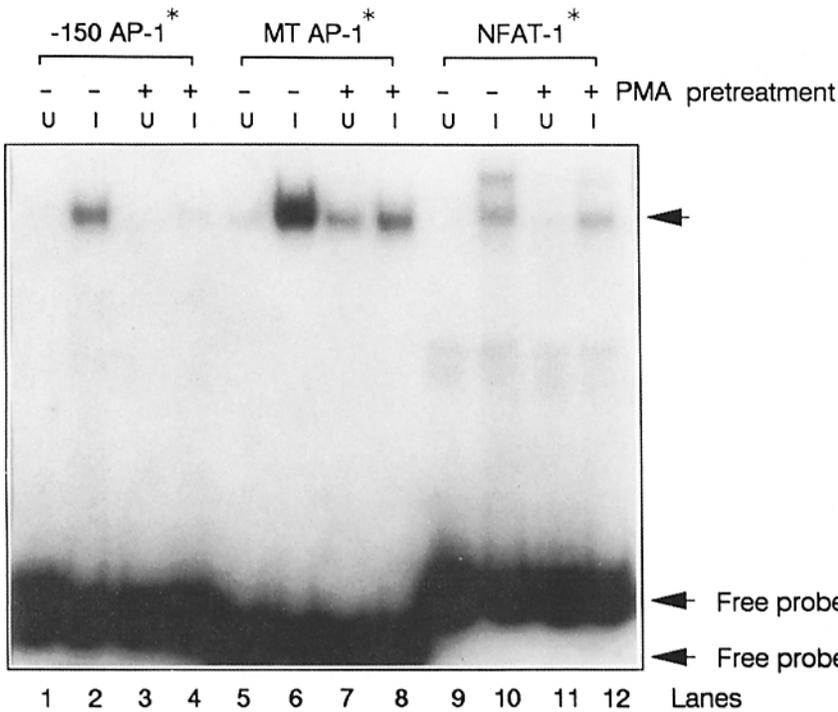


Figure 3. Site-specific mutations in the downstream AP-1 site markedly decrease induction of the IL-2 promoter in vivo. (a) Induction of CAT activity from wild-type (lanes 2 and 3) and mutant (lanes 4–7) IL-2 promoters after transfection into Ar-5 cells. Lane 1 shows the activity obtained after transfection of the control pRSVCAT plasmid. Lanes 1 and 2, unstimulated cells; lanes 3–7, cells stimulated with crosslinked anti-CD3 ϵ . One representative experiment, of six to eight using different methods of activation, is shown. There was no difference in the background activity of the wild-type and mutant promoters in unstimulated cells. (Top) Sequences of the mutations in the two AP-1 sites of the IL-2 promoter. (b) PKC-sensitive binding of a nuclear factor from activated Ar-5 cells to the downstream AP-1 site of the IL-2 promoter. Cells were untreated (“–”; lanes 1, 2, 5, 6, 9, and 10) or depleted of kinase C by prolonged exposure to the phorbol ester PMA (“+”; lanes 3, 4, 7, 8, 11, and 12), and left unstimulated (“U”; lanes 1, 3, 5, 7, 9, and 11), or induced with crosslinked anti-CD3 (“I”; lanes 2, 4, 6, 8, 10, and 12). Nuclear extracts were made, incubated with labeled oligonucleotides corresponding to the –150 AP-1 site (lanes 1–4), the metallothionein AP-1 site (lanes 5–8), or the NFAT-1 site (lanes 9–12), and analyzed by electrophoretic mobility shift assay. The positions of the free probes and of the inducible retarded complexes are indicated.

for negative regulation in Ar-5 cells. In contrast, the same NF- κ B mutations have been reported to decrease induction in PHA/PMA-stimulated JURKAT cells by as much as 80% (13). This discrepancy may reflect a difference between primary and transformed T cells. Alternatively, stimulation via PMA and PHA may not physiologically mimic activation by antigen or antibodies to the TCR/CD3 complex. Nevertheless, Ar-5 cells and JURKAT cells are unlikely to have completely different requirements for IL-2 promoter activation, since a mutant promoter with an internal deletion encompassing the NF- κ B site was induced as well as the wild-type IL-2 promoter in PHA/PMA-stimulated JURKAT cells (12, 48).

Like induction of the wild-type promoter, induction of the IL-2 κ B site mutants was also abrogated by PMA pretreatment (Fig. 2 *a*, lanes 5, 6; 9, 10; 13, 14; and 17, 18). This result confirms that the NF- κ B site does not represent the PKC-responsive site in the IL-2 promoter. The IL-2 κ B site binds purified NF- κ B in vitro with \sim 10-fold lower affinity than the Ig κ B site (49), and its activity in vivo may be further reduced or modified by interactions with other nuclear factors (47, 50); thus, it is not surprising that it appears non-functional in our assays.

Although mutations in the NF- κ B site did not alter the induction of IL-2-CAT activity, we detected binding of an inducible nuclear factor to the NF- κ B site in gel-shift assays (Fig. 2 *b*). A double-stranded oligonucleotide spanning the murine IL-2 κ B site (-210 to -186 bp) was labeled and incubated with nuclear extracts from Ar-5 cells that were either unstimulated or stimulated with crosslinked anti-CD3 ϵ . Two specific retarded bands were observed with nuclear extracts from stimulated, but not unstimulated cells (Fig. 2 *b*, lanes 1 and 2). The upper band migrated with the same mobility as the retarded bands seen with a labeled (Ig) κ enhancer κ B probe (data not shown). The lower and fainter band appeared variably and may result from proteolytic degradation of the upper band. The binding was competed by unlabeled oligonucleotides corresponding to the labeled IL-2 κ B probe (Fig. 2 *b*, lanes 3 and 4, "self"), as well as by oligonucleotides containing the Ig κ B site (Fig. 2 *b*, lanes 5 and 6). Ig κ B oligonucleotides containing mutations in the 5' invariant GGG (similar to the M1 mutation in IL-2 κ B, lanes 7 and 8 "5' Mut") or in the 3' TTCC (similar to the M4 mutation in IL-2 κ B, lanes 9 and 10, "3' Mut") failed to compete for binding (Fig. 2 *b*). The factor therefore, behaved as predicted for authentic NF- κ B (13, 42). The discrepancy between the binding seen in the gel-shift experiments and the lack of effect in transfection assays emphasizes the importance of corroborating in vitro binding analyses with experiments performed in vivo.

Mutations in the Downstream AP-1 Site Abrogate IL-2 Promoter Induction. We also investigated the role of both AP-1 sites in induction of the IL-2 promoter. Two AP-1 sites have been identified by deletion and footprinting analysis at -150 and -180 bp upstream of the transcription start site (12, 14, 15, 30) (Figs. 1 *a* and 3 *a*). The upstream AP-1 site at -180 bp is not detected by footprinting of EL-4 nuclear extracts

(14), and deletion of the site does not affect induction of the IL-2 promoter in JURKAT cells (12). However, this site has been implicated in the synergistic response to IL-1 of LBRM-33 cells (16). By contrast, deletion of the downstream AP-1 site at -150 bp decreases induction to 11% of the wild-type level in JURKAT cells, and to 27% of the wild-type level in EL-4 cells (12, 14). This site binds inducible nuclear proteins from EL-4 cells, and has a higher affinity for purified AP-1 proteins than the -180 bp site (14). We made site-specific mutations in both AP-1 sites and transfected the mutants into Ar-5 cells (Fig. 3, *a* and *b*).

Both mutations in the downstream (-150 bp) AP-1 site significantly decreased IL-2 promoter activity in transfection experiments. Mutant M3, bearing a triple base mutation (AAA to CTC) showed a \sim 95% decrease in the induction of CAT activity in response to crosslinked anti-CD3 ϵ (Fig. 3 *a*, bottom), and a 60–80% decrease in response to TA3 cells and anti-CD3 ϵ or PMA and ionomycin (data not shown). If normalized with respect to wild-type plasmid for transfection efficiency, induction of the mutant M3 promoter would be decreased by two to threefold further (see normalized hGH values in Materials and Methods). Mutant M4, which also bears a triple mutation (AGT to CAA) showed no response at all to any stimulus tested (>95% decrease, Fig. 3 *a*, bottom, and data not shown), although the plasmid was transfected better than the wild-type. In contrast the M1 and M2 mutations in the upstream AP-1 site only partly decreased induction in response to these various stimuli (25–65% decrease, Fig. 3 *a*, bottom, and data not shown). These results indicate that the downstream AP-1 site is absolutely required for induction of the IL-2 promoter in response to TCR stimuli. The upstream site, although not critical, may enhance activation in vivo. Our mutations monitor the AP-1 site at -150 bp rather than the CD28-responsive site that is located immediately upstream (19), since they abrogate induction of CAT activity in cells stimulated with crosslinked anti-CD3 in the complete absence of accessory cells that might express ligands for CD28.

We performed gel-shift assays to determine if either AP-1 site was capable of binding in a PKC-sensitive manner to inducible nuclear factors in vitro. Radiolabeled oligonucleotides corresponding to either AP-1 site were incubated with nuclear extracts from stimulated or unstimulated Ar-5 cells. The -180 AP-1 site probe failed to bind any strongly inducible protein (data not shown). These results are in contrast to those reported for LBRM cells (16), and may reflect the inability of IL-1 to enhance IL-2 gene induction in Ar-5 cells (Jain, J., and A. Rao, unpublished results). In contrast, the oligonucleotide corresponding to the -150 AP-1 site bound to a nuclear factor found only in activated Ar-5 cells (Fig. 3 *b*, lanes 1 and 2); an inducible factor of identical mobility bound to an oligonucleotide containing the metallothionein AP-1 site (Fig. 3 *b*, lanes 6 and 7). The factor was identified as Fos or a Fos-related protein (30). It was detectable at 30 min after activation and was maximally induced at 1–2 h, as expected if the rapidly inducible Fos family members were involved. Its migration was altered by affinity-purified anti-

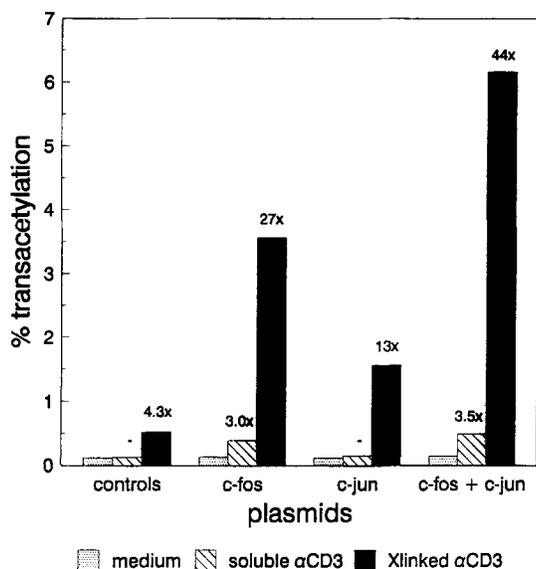


Figure 4. Induction of the IL-2 promoter is augmented by expression of c-Fos and c-Jun. Ar-5 cells were cotransfected with 2.5 μ g of the human 5' IL-2-CAT plasmid and 2.5 μ g each of the human c-Fos, c-Jun, or control expression plasmids. The cells were activated with 1% anti-CD3, either soluble (hatched bars), or crosslinked with immobilized second antibody (solid bars). One representative experiment, of three similar ones, is shown.

bodies to the Fos M peptide (51) that were capable of immunoprecipitating c-Fos and related proteins. Its binding was competed by unlabeled oligonucleotides corresponding to the downstream AP-1 site or the metallothionein AP-1 site, but not by oligonucleotides containing the IL-2 κ B site or the M1, M2, M3, or M4 mutations in the AP-1 sites (30). Induction of the AP-1 factor in Ar-5 cells was sensitive to PKC depletion by chronic phorbol ester treatment (Fig. 3 b; compare lanes 2 and 4 with 6 and 8), although there was no effect on induction of NFAT-1 in the same experiment (Fig. 3 b, lanes 9–12).

Cotransfection with c-Fos and c-Jun Expression Plasmids Increases IL-2 Promoter Induction. Fos-Jun complexes are induced after TCR/CD3-mediated activation (16, 20) and bind to AP-1 sites in vitro (7). We tested the ability of c-Fos and c-Jun expression plasmids to enhance the induction of IL-2-CAT activity in transient cotransfections (Fig. 4). As high concentrations of plasmid DNA (>10 μ g) inhibit transfection efficiency in these cells (Valge-Archer, V. E., and A. Rao, unpublished results), the amount of IL-2-CAT plasmid transfected was decreased by the amount of c-Fos, c-Jun, or con-

trol plasmids used. Under these conditions, the IL-2-CAT plasmid cotransfected with control plasmids for c-Fos and c-Jun was weakly induced by crosslinked anti-CD3 ϵ (4.3-fold in this experiment), presumably because the FBJ-MSV and RSV LTRs present in the control plasmids competed for binding of transcription factors also required for IL-2 promoter induction (see Materials and Methods). Cotransfection of either c-Fos or c-Jun with IL-2-CAT resulted in increased induction of CAT activity in stimulated T cells (to 27-fold and 13-fold, respectively; Fig. 4). Cotransfection of both c-Fos and c-Jun with IL-2-CAT resulted in a 44-fold induction of IL-2-CAT. This represents a 10-fold enhancement over the control cotransfection (Fig. 4). In addition, cotransfection of either c-Fos alone, or c-Fos and c-Jun together uncovered a threefold induction of IL-2-CAT by soluble anti-CD3 ϵ , which alone was insufficient to induce IL-2-CAT expression.

Cotransfection of c-Fos and c-Jun, either individually or together, did not cause induction of IL-2-CAT activity in unstimulated T cells, confirming that other transcription factors such as NFAT-1 are also required for induction. Cotransfection with c-Fos alone causes a reproducible three- to fivefold increase in the induction of IL-2-CAT activity in stimulated T cells, possibly because induction of c-Jun (which is expressed in resting cells) is not limiting for AP-1 activity (30). In contrast, cotransfection with c-Jun alone has a more variable effect, indicating that induction of c-Fos or other Fos family members may be limiting. Cotransfection with c-Fos did not increase induction of the AP-1 site mutants M3 and M4 in response to crosslinked anti-CD3 ϵ (30). These results are consistent with the possibility that c-Fos and c-Jun act to enhance IL-2-CAT induction by binding to the -150 bp AP-1 site, and provide further in vivo evidence for the importance of the -150 AP-1 site for IL-2 promoter activation in untransformed T cells.

Conclusions. In summary, we have demonstrated that the IL-2 NF- κ B site is not required for TCR-mediated induction of the IL-2 promoter in an untransformed T cell clone. In contrast, the AP-1 site at -150 is clearly required for induction in vivo. In the absence of any inducible binding with the -180 AP-1 site in gel-shift assays, our results establish that the -150 AP-1 site is a major, if not the only, kinase C-responsive site in the IL-2 promoter. Cotransfection with c-fos and c-jun expression plasmids enhances IL-2 promoter induction, most likely by acting at the AP-1 site at -150 bp. However, it remains to be established which of the various Fos/Jun family members actually mediate activation of the IL-2 promoter in vivo.

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