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Calcineurin Potentiates Activation of the Granulocyte-Macrophage Colony-stimulating Factor Gene in T Cells: Involvement of the Conserved Lymphokine Element 0

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2) are produced by stimulation with phorbol-12-myristate acetate (PMA) and calcium ionophore (A23187) in human T cell leukemia Jurkat cells. The expression of GM-CSF and IL-2 is inhibited by immunosuppressive drugs such as cyclosporin A (CsA) and FK506. Earlier studies on the IL-2 gene expression showed that overexpression of calcineurin (CN), a Ca^{2+}/calmodulin-dependent protein phosphatase, can stimulate transcription from the IL-2 promoter through the NF-AT-binding site. In this study, we obtained evidence that transfection of the cDNAs for CN A (catalytic) and CN B (regulatory) subunits also augments transcription from the GM-CSF promoter and recovers the transcription inhibited by CsA. The constitutively active type of the CN A subunit, which lacks the auto-inhibitory and calmodulin-binding domains, acts in synergy with PMA to activate transcription from the GM-CSF promoter. We also found that the active CN partially replaces calcium ionophore in synergy with PMA to induce expression of endogenous GM-CSF and IL-2. By multimerizing the regulatory elements of the GM-CSF promoter, we found that one of the target sites for the CN action is the conserved lymphokine element 0 (CLE0), located at positions between −54 and −40. Mobility shift assays showed that the CLE0 sequence has an AP1-binding site and is associated with an NF-AT-like factor, termed NF-CLE0 γ. NF-CLE0 γ binding is induced by PMA/A23187 and is inhibited by treatment with CsA. These results suggest that CN is involved in the coordinated induction of the GM-CSF and IL-2 genes and that the CLE0 sequence of the GM-CSF gene is a functional analogue of the NF-AT-binding site in the IL-2 promoter, which mediates signals downstream of T cell activation.

INTRODUCTION

Activation of T cells by recognition of antigens on antigen-presenting cells leads to induction of nuclear proto-oncogenes such as *c-fos* in 15 to 30 min, followed by induction of a battery of lymphokine genes, including the interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF) genes (Arai *et al.*, 1990; Ullman *et al.*, 1990). Upon T cell activation, one of the major events is hydrolysis of phosphatidylinositol-4,5-biphosphate to generate inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DG) by phospholipase C (Berridge, 1993). IP_3 raises the cytoplasmic Ca^{2+} concentration from sequestered intracellular stores, thereby opening Ca^{2+} channels in the plasma membrane (Berridge, 1993). This process can be functionally mimicked by a Ca^{2+} ionophore such as A23187. DG together with Ca^{2+} activates protein kinase C (PKC), and phorbol esters such as phorbol-12-myristate acetate (PMA) can mimic the effect of DG. Stimulation with PMA and Ca^{2+}

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ionophore leads to considerable induction of several lymphokine genes in T cells (Arai *et al.*, 1992). In particular, there is strong synergy between PKC- and Ca^{2+}-signaling pathways in the production of GM-CSF or IL-2 (Arai *et al.*, 1992; Riegel *et al.*, 1992). The expression of GM-CSF and IL-2 is inhibited by immunosuppressive drugs such as cyclosporin A (CsA) and FK506 (Arai *et al.*, 1992; Riegel *et al.*, 1992).

Earlier studies on IL-2 gene expression revealed that a nuclear factor of activated T cells (NF-AT) plays a crucial role in transcriptional activation and is one of the functional targets for CsA and FK506 (Riegel *et al.*, 1992). NF-AT appears to consist of two factors: one is a ubiquitous nuclear factor ($NF\text{-}AT_n$), induced through the PKC pathway and identified as AP-1 (Jun- and Fos-family proteins) (Jain *et al.*, 1992). The other is a T cell-specific, pre-existing component ($NF\text{-}AT_c$), and translocation from cytoplasm to nucleus is regulated through the Ca^{2+} pathway and inhibited by CsA and FK506 (Flanagan *et al.*, 1991). These drugs bind endogenous intracellular receptors (immunophilins) and the resulting complexes target a Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase, calcineurin (CN), to exert immunosuppressive effects (Schreiber and Crabtree, 1992). CN is composed of catalytic (A) and regulatory (B) subunits, and overexpression can restore the NF-AT-dependent transcription inhibited by CsA or FK506 in T cells (Clipstone and Crabtree, 1992). In addition, a constitutively active form of the CN A subunit acts in synergy with PMA to activate transcription of the IL-2 gene through the NF-AT-binding site, thereby indicating that expression of activated CN can mimic a Ca^{2+} signaling pathway in T cells (O'Keefe *et al.*, 1992).

We have identified two elements, GM-κB/GC box (formerly GM2/GC box) and CLE0, as essential *cis*-elements of the mouse GM-CSF gene (Miyatake *et al.*, 1988b, 1991; Heike *et al.*, 1989). Together these elements are responsible for maximal activation, induced by costimulation with PMA/A23187 in T cells (Miyatake *et al.*, 1988b, 1991; Heike *et al.*, 1989). The GM-κB/GC-box sequence between −95 and −73 contains two DNA-binding motifs: one (GGTAGTTCCC) is recognized by an inducible factor, NF-GM2 and the other (CCGCCC) by a set of constitutive factors, A1 (Sp1), A2, and B (Sugimoto *et al.*, 1990). Purification of NF-GM2 from nuclear extracts of PMA/A23187-stimulated Jurkat cells revealed that the GM-κB sequence is recognized by NF-GM2 consisting of 50- and 65-kDa polypeptides which are immunologically indistinguishable from 50- and 65-kDa subunits of NF-κB, respectively (Tsuboi *et al.*, 1991). Mutations in either GM-κB or GC-box sequence abolish the inducibility of the GM-CSF gene as well as the DNA binding of respective factors (Sugimoto *et al.*, 1990).

The other element, conserved lymphokine element 0 (CLE0), extends from −54 to −40 downstream of the GM-κB/GC-box element and shares strong homology with the 5'-flanking regions of the IL-4 and IL-5 genes (Miyatake *et al.*, 1991). Mutations in the CLE0 element fail to induce transcription from the GM-CSF gene as well as DNA binding to this sequence in response to PMA/A23187 (Miyatake *et al.*, 1991). We report here that overexpression of CN augments transcriptional activation of the GM-CSF gene, presumably through the CLE0 element. Moreover, the CLE0 element is associated with an NF-AT-like factor, whose binding is induced by PMA/A23187 and inhibited by addition of CsA. The coordinated regulation of the GM-CSF and IL-2 genes through the CLE0-like element in T cells is discussed.

MATERIALS AND METHODS

Cell Culture and Lymphokine Assays

Jurkat cells (a human T cell leukemia line), provided by Dr. P. Berg (Stanford University, Stanford, CA), were maintained in RPMI 1640 medium (JRH Biosciences) containing 10% fetal calf serum, penicillin G (100 U/ml), and streptomycin (100 mg/ml). Cells at a density of 2×10^6 cells/ml were treated with phorbol 12-myristate 13-acetate (PMA) (0–50 ng/ml, Calbiochem, La Jolla, CA) and A23187 (0–2.5 μM, Calbiochem) in the presence of CsA (Sandimmun, Sandoz, Basel, Switzerland) at the concentration of 0 to 1.0 μg/ml. After incubation for 24 h, the supernatant was collected by centrifugation and subjected to ELISA for GM-CSF and IL-2 (Abrams *et al.*, 1992).

Mobility Shift Assays

Nuclear extracts were prepared on a small scale, basically according to the method of Schreiber *et al.* (1989) after treatment of Jurkat cells ($2–5 \times 10^7$) with 50 ng/ml PMA, 1 μM A23187, or both in the presence or absence of 1 μg/ml CsA for 2–3 h (Masuda *et al.*, 1993). In binding assays to the GC-box (gm-mu 7.8) and GM-κB (gm-mu 17.18) sequences, binding reaction and electrophoresis were done using 2.5 μg and 10 μg of nuclear extracts, respectively, as described elsewhere (Tsuboi *et al.*, 1991). The binding reaction to the CLE0 and NF-AT sequences was carried out in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic (HEPES)-NaOH (pH 7.9), 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol (DTT), 50 μg/ml poly(dI-dC)(Pharmacia), 250 μg/ml bovine serum albumin (BSA), 100 mM KCl, ~0.5 ng of labeled probe (~50 000 cpm), and 12 μg of nuclear extracts. After incubation for 30 min at room temperature, the sample was applied onto a 4% native polyacrylamide gel in a tris(hydroxymethyl)aminomethane (Tris)-glycine-EDTA buffer. The gel was dried and autoradiographed after electrophoresis at 120 V at room temperature.

Oligonucleotides for mobility-shift and competition assays are shown below as one strand, with the overhang in lower case. Underlines depict a substitution mutation as described elsewhere (Sugimoto *et al.*, 1990).

GC-box (gm-mu 7.8): gatcAGGTAGTGCCCCCGCCCCCC (Sugimoto *et al.*, 1990)

GM-κB (gm-mu 17.18): gatcAGGTAGTTCCCCCGCACCCC (Sugimoto *et al.*, 1990)

CLE0: gatcGTCACCATTAATCATTTCCTCTAACTGT (Miyatake *et al.*, 1991)

NF-AT: gatcGGAGGAAAAACTGTTTCATACAGAAGGCGT (Emmel *et al.*, 1989)

Sp1: GATCGATCGGGGCGGGGCGATC (Dynan and Tjian, 1983)

Plasmid Construction

All plasmids were constructed and manipulated using standard techniques (Sambrook *et al.*, 1989). All DNA sequences inserted into vectors

were confirmed by the dideoxy chain termination method using Sequenase (United States Biochemicals, Cleveland, OH). pIL-2 CAT-1, provided from Dr. G.R. Crabtree (Stanford), has sequences −567 to +47 from the human IL-2 gene controlling expression of a chloramphenicol acetyltransferase (CAT) gene (Siebenlist *et al.*, 1986). pIL-2 Luc-1 was constructed by replacing the SRα promoter (*Hind*III fragment) of pSRα-L-AΔ5′ (Takebe *et al.*, 1988) with the IL-2 promoter (*Hind*III fragment) of pIL-2 CAT-1.

pKC1 carrying a sequence −97 to +27 of the mouse GM-CSF gene linked to the CAT gene was constructed by replacing the *Bgl*II–*Bst*EII fragment of pmGMCAT-740 (Miyatake *et al.*, 1988b) with synthetic oligonucleotides (N1:N2).

N1:N2
gatcAACTCAGGTAGTTCCCCCGCCCCCCTGGAGTTCTGTG
TTGAGTCCATCAAGGGGGCGGGGGGACCTCAAGA-
CACCAGTG

pKC17 harboring a sequence −97 to +27 of the GM-CSF gene linked to the luciferase gene was constructed by replacing the *Nde*I–*Sac*I fragment of pmGMLuc-740 (Heike *et al.*, 1989) with the *Nde*I–*Sac*I fragment of pKC1.

p(GM-κB/GC-box)$_2$ CAT and p(CLE0)$_2$ CAT (Figure 6B) were constructed by replacing the *Bgl*II–*Sac*I fragment of pmGMCAT-740 with synthetic oligonucleotides, which contain two copies of the GM-κB/GC-box (−91 to −72) and CLE0 (−70 to −37) sequences, respectively. p(CLE0)$_3$ CAT (Figure 6D) was also generated by replacing the *Bgl*II–*Sac*I fragment of pmGMCAT-740 with synthetic oligonucleotides, which contain three copies of the CLE0 sequence −57 to −37 linked by *Bgl*II and *Sac*I overhangs.

pBJ5-CNA and pBJ5-CNB, provided by Dr. N.A. Clipstone (Stanford), contain murine cDNAs for the $A_{\alpha 1}$ and B subunits of CN, respectively, under control of the SRα promoter (Clipstone and Crabtree, 1992). A deletion mutant of the CN catalytic subunit (pBJ5-CNΔA), which lacks the putative auto-inhibitory and calmodulin-binding domains in the C-terminus, was also constructed by Dr. Clipstone.

Transfection, CAT and Luciferase Assays

Transient transfection assay was done using the DEAE-dextran method (Miyatake *et al.*, 1988a), except for the experiments of lymphokine production. Jurkat cells (2×10^7) washed with Tris-buffered saline were suspended in 3 ml of the same buffer containing DEAE-dextran (0.5 mg/ml, Pharmacia, Piscataway, NJ) and 30–36 μg of total plasmid DNA, incubated for 20 min at room temperature, and mixed with 30 ml of culture medium containing 0.1 mM chloroquine diphosphate (Sigma, St. Louis, MO). After incubation for 1 h at 37°C, chloroquine-containing medium was replaced with 20 ml of culture medium. The cell suspension was divided into four wells of a 6-well plate, cultured for 30 h, and treated with 50 ng/ml PMA and 1 μM A23187 in the presence of 0, 0.01 or 1 μg/ml CsA for 12–15 h. Cell extracts were prepared by four cycles of freezing and thawing, and subjected to CAT and luciferase assays.

CAT activity was measured by an AMBIS image analyzer as percentage conversion of [^{14}C]chloramphenicol to 1- or 3-acetylated chloramphenicol after 6 h incubation at 37°C with 50 μg of cell extracts, as described previously (Miyatake *et al.*, 1988a).

Luciferase activity was measured by a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA) with 10 μl of 50 μl cell extracts, followed by division of a value by a protein amount of 10 μl extracts, as described previously (de Wet *et al.*, 1987).

Jurkat cells were transfected by electroporation (Gene Pulsar, Bio-Rad, Richmond, CA) to measure lymphokine production in these cells. Cells (4×10^7) in 0.5 ml RPMI medium were mixed with 100 μg of DNA in 0.4 cm cuvettes (Bio-Rad), pulsed at 300 V (960 μF), then left at room temperature for 10 min before addition of 10 ml of culture medium after which the mixture was transferred to plates. After 36 h of incubation, the cells were harvested, suspended in 4 ml of culture medium, divided into four wells of 16-well plates and treated with various reagents for 24 h.

RESULTS

CsA Blocks GM-CSF Production in Jurkat Cells

Jurkat T cells produced GM-CSF when co-stimulated with PMA and A23187 (Figure 1A). The production was maximal when the cells were treated with 50 ng/ml PMA and 1.0 μM A23187. Either PMA or A23187 did not induce the expression of GM-CSF. The GM-CSF production, induced by PMA/A23187, was inhibited in the presence of the immunosuppressive drug CsA, in a dose-dependent manner (Figure 1B). Likewise,

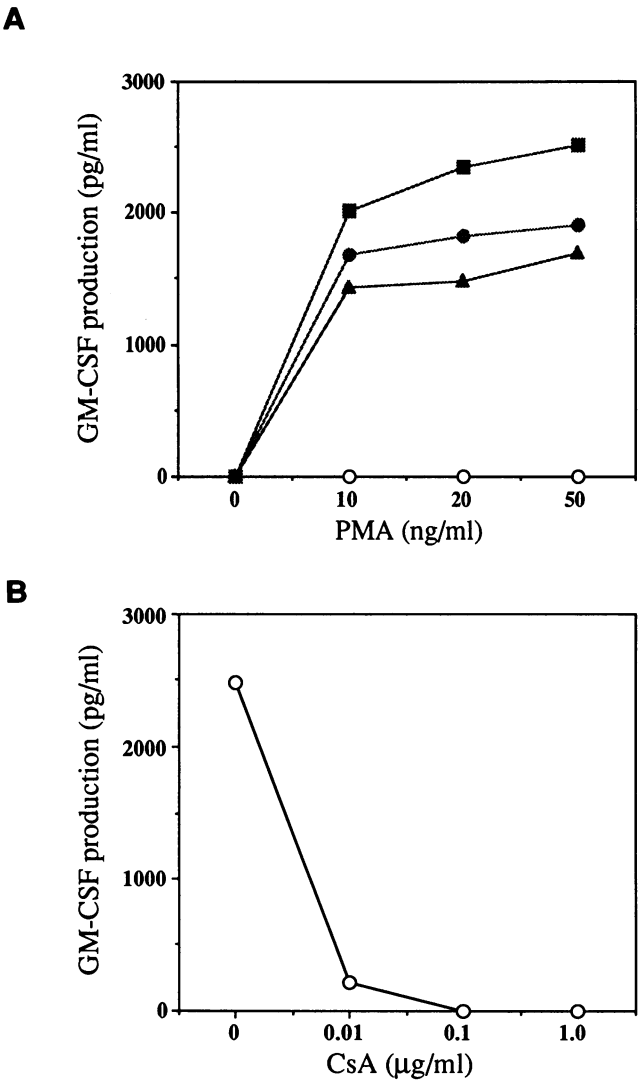

Figure 1. CsA blocks GM-CSF production induced by costimulation with PMA and A23187 in Jurkat cells. (A) Jurkat cells were stimulated with 0, 10, 20, and 50 ng/ml of PMA in the presence of A23187: 0 μM (○), 0.5 μM (●), 1.0 μM (■) and 2.5 μM (▲). GM-CSF was measured by ELISA after 24 h incubation. (B) Jurkat cells were treated for 24 h with PMA (50 ng/ml) and A23187 (1.0 μM) in the presence of CsA (0, 0.01, 0.1, or 1.0 μg/ml). Results shown are the averages of 3 experiments.

IL-2 production was induced by co-stimulation with PMA/A23187 and was blocked by CsA treatment of Jurkat cells. These results show strong synergy between PKC- and Ca^{2+}-signaling pathways for the production of GM-CSF and IL-2, which is completely inhibited by addition of CsA.

CLE0 Motif Is Recognized by a CsA-sensitive NF-AT-like Factor

Mobility shift assays using nuclear extracts of Jurkat cells were performed to identify PMA/A23187-induced nuclear factor(s), whose binding is inhibited by the addition of CsA, in the GM-CSF promoter. A1, A2, and B bindings to the GC-box sequence (gm-mu 7.8) were constitutive and were not affected significantly by any treatment given (Figure 2A). NF-κB binding to the GM-κB sequence (gm-mu 17.18) was significantly induced by PMA alone. PMA/A23187 treatment slightly increased the NF-κB binding activity (<1.5-fold), and CsA treatment inhibited the PMA/A23187-induced binding, to the PMA-induced level (Figure 2A).

We found in earlier mobility shift assays that the CLE0 sequence gives rise to at least three DNA-protein complexes, termed NF-CLE0 α, β, and γ (Masuda *et al.*, 1993). A basal level of NF-CLE0 α and β was detected in the non-stimulated nuclear extracts and were induced markedly by PMA (Figure 2B). PMA/A23187 treatment further increased the α- and β-binding activities less than threefold, and the addition of CsA was without effect (Figure 2B). Several lines of evidence including the binding of partially purified AP-1 to CLE0 sequence indicated that these are related to AP-1 (Masuda *et al.*, 1993). In contrast, NF-CLE0 γ was not induced by PMA or A23187 alone, but was induced by PMA/A23187 (Figure 2B). This binding was completely blocked by adding CsA. Interestingly, the NF-AT-binding sequence competitively inhibited NF-CLE0 γ binding, but not α and β bindings (Figure 2C). These results suggest that the CLE0 element of the GM-CSF gene is one of the targets for the NF-AT-related factor, which is induced by co-stimulation with PMA/A23187 and blocked by CsA treatment.

Overexpression of CN Potentiates Transcriptional Activation from the GM-CSF Promoter in Jurkat Cells

Since CN was shown to be implicated in the Ca^{2+}-signaling pathway of the IL-2 gene induction (Clipstone and Crabtree, 1992; O'Keefe *et al.*, 1992), we tested the effect of CN on transcription from the GM-CSF promoter. pBJ5-CNA and pBJ5-CNB carry cDNAs for the catalytic (A) and regulatory (B) subunits of CN, respectively (Clipstone and Crabtree, 1992). pKC1 is a CAT reporter construct carrying the GM-CSF promoter between positions −97 and +27. First we co-transfected pKC1 with a control vector pBJ5 into Jurkat cells. PMA alone slightly stimulated transcription from the GM-CSF promoter, while A23187 alone did not. PMA/A23187 treatment synergistically induced transcription from the GM-CSF promoter, and this induction was inhibited by CsA, in a dose-dependent fashion (Figure 3A). Co-transfection of pKC1 with pBJ5-CNA and pBJ5-

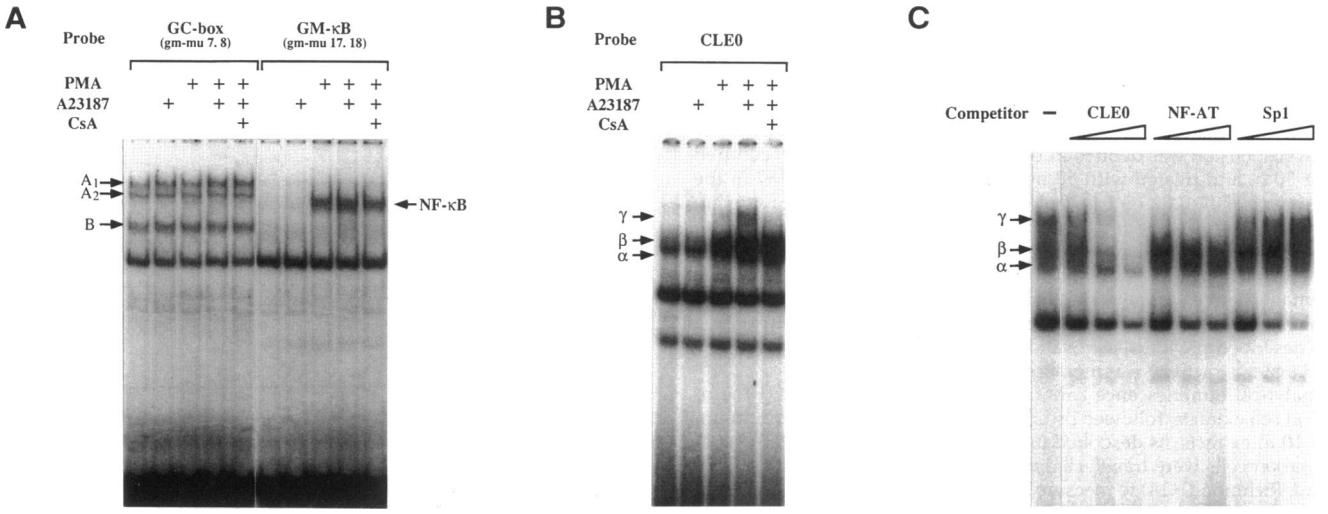

Figure 2. CLE0 element is recognized by an NF-AT-like factor whose binding is induced by PMA/A23187 and inhibited by CsA. Nuclear extracts from either non-A23187-, PMA-, PMA/A23187-, or PMA/A23187/CsA-stimulated Jurkat cells were incubated with either GC-box (A), GM-κB (A) or CLE0 (B) oligonucleotides labeled with ^{32}P, followed by native acrylamide gel electrophoresis and autoradiography. (C) Nuclear extracts of PMA/A23187-stimulated cells were incubated with ^{32}P-labeled CLE0 oligonucleotides together with a competitor of either CLE0, NF-AT or Sp1 oligonucleotides at the concentration of 0.5, 5, and 50 ng. The following amounts of nuclear extracts were used for assays: GC-box (A), 2.5 μg, GM-κB (A), 10 μg, (B), 12 μg, and (C), 12 μg.

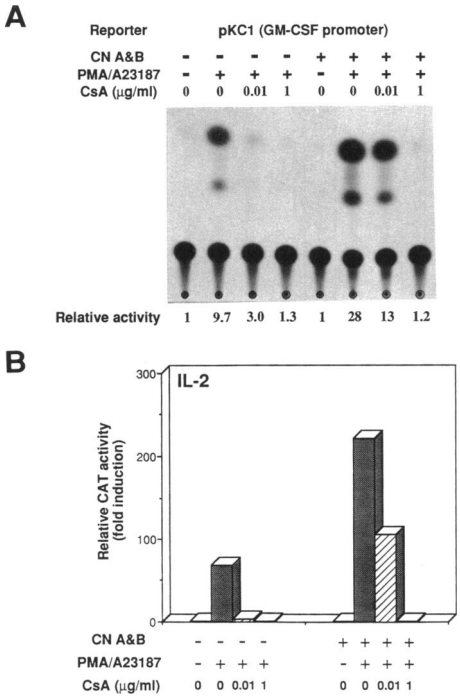

Figure 3. Exogenously expressed CN in Jurkat cells stimulates transcription from the GM-CSF and IL-2 promoters and restores the transcription inhibited by CsA. Jurkat cells (2×10^7) were co-transfected either with 12 μg of pKC1 (A) or pIL-2 CAT-1 (B) together with either pBJ5 (24 μg) or pBJ5-CNA (12 μg) and pBJ5-CNB (12 μg), and divided into 4 aliquots. After 30 h incubation, the cells were stimulated for 15 h with PMA (50 ng/ml) and A23187 (1 μM) in the presence of CsA (0, 0.01, or 1.0 μg/ml). Results are shown as thin layer chromatogram (A), quantitated as fold induction of CAT activity compared with unstimulated cells (A and B), and are the averages of 3 independent experiments. Means and standard deviations (SD) are 1 ± 0.34, 9.7 ± 1.6, 3.0 ± 1.2, 1.3 ± 0.14, 1 ± 0.065, 28 ± 5.7, 13 ± 2.3, and 1.2 ± 0.26 (A); and 1 ± 0.50, 69 ± 11, 3.5 ± 4.4, 0.94 ± 0.20, 1 ± 0.24, 220 ± 30, 110 ± 18, and 1.2 ± 0.32 (B).

CNB enhanced transcription from the GM-CSF promoter threefold and recovered the transcription inhibited by 0.01 μg/ml of CsA, compared with pBJ5 (Figure 3A). As a control experiment, we co-transfected pIL-2 CAT-1 (−567 to +47) along with pBJ5-CNA and pBJ5-CNB into Jurkat cells. Overexpression of CN augmented transcription from the IL-2 promoter more than threefold and restored the transcription reduced by CsA in the concentration of 0.01 μg/ml, compared with pBJ5 (Figure 3B). These results indicate that overexpressed CN potentiates transcriptional activation of the GM-CSF gene in response to PMA/A23187 and confers CsA resistance on the transcription.

Expression of Constitutively Active CN Acts in Synergy with PMA to Facilitate Transcription from the GM-CSF Promoter

To further examine the CN action on the GM-CSF promoter, we carried out co-transfection experiments using a deletion mutant of the CN catalytic subunit (pBJ5-CNΔA). This mutation, which lacks the putative autoinhibitory and calmodulin-binding domains, gives rise to Ca^{2+}/calmodulin-independent, constitutive phosphatase activity (Clipstone, personal communication). pKC17 is a luciferase reporter construct containing the GM-CSF promoter between −97 and +27. First we co-transfected pKC17 with the control vector pBJ5 into Jurkat cells. PMA alone slightly stimulated transcription from the GM-CSF promoter, and this activation was not blocked by addition of CsA (Figure 4A). Co-trans-

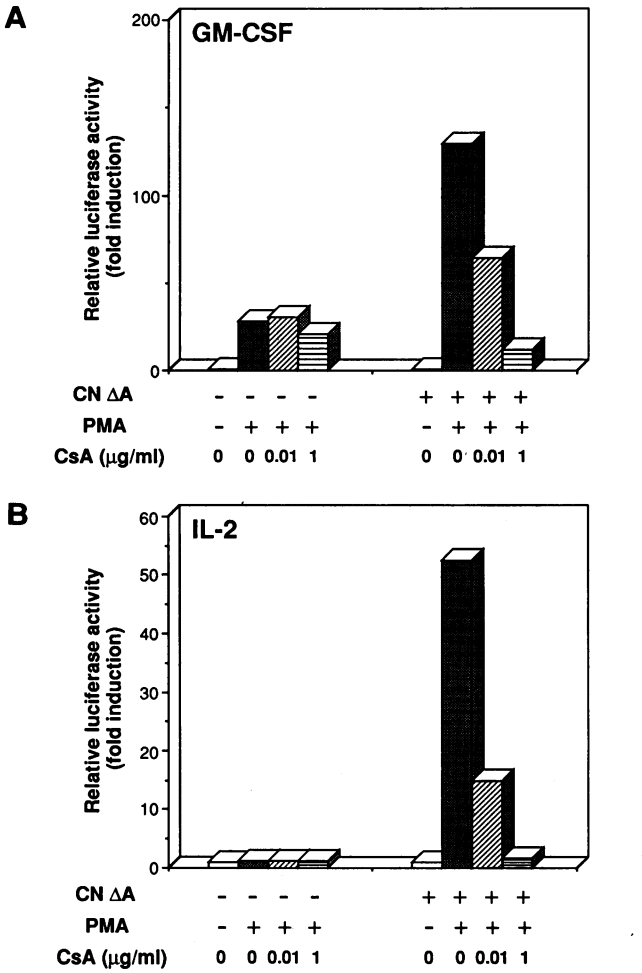

Figure 4. Expression of constitutively active CN synergizes with PMA to activate transcription from the GM-CSF and IL-2 promoters in Jurkat cells. Cells (2×10^7) were co-transfected either with 10 μg of pKC17 (A) or pIL-2 Luc-1 (B) along with either pBJ5 (20 μg) or pBJ5-CNΔA (20 μg) and divided into 4 aliquots. After 30 h incubation, the cells were stimulated for 12 h with PMA (50 ng/ml) in the presence of CsA (0, 0.01, or 1.0 μg/ml). Results presented are quantitated as fold induction of luciferase activity compared with unstimulated cells and are the averages of 3 independent experiments. Means and SD are 1 ± 0.24, 28 ± 9.5, 30 ± 7.0, 21 ± 7.2, 1 ± 0.44, 130 ± 32, 64 ± 16 and 12 ± 4.2 (A), and 1 ± 0.28, 1.3 ± 0.070, 1.2 ± 0.36, 1.2 ± 0.15, 1 ± 0.42, 53 ± 23, 15 ± 6.2 and 1.6 ± 0.17 (B).

fection of pKC17 with pBJ5-CNΔA enhanced transcription from the GM-CSF promoter 4.5-fold and rendered the cells more resistant to the effect of CsA (0.01 μg/ml), compared with pBJ5 (Figure 4A). As a control experiment, we co-transfected pIL-2 Luc-1 (−567 to +47) along with pBJ5-CNΔA into Jurkat cells. Expression of the activated CN stimulated transcription from the IL-2 promoter 40-fold and also made the cells more resistant to the effect of CsA (0.01 μg/ml), compared with pBJ5 (Figure 4B). It would thus appear that the region between −97 and +27 of the GM-CSF promoter is responsible for the synergistic action of the activated CN with PMA.

Constitutively Active CN Acts in Synergy with PMA to Induce Production of Endogenous GM-CSF and IL-2

To determine whether increases in luciferase activity by the synergistic action of the constitutively active CN with PMA were indeed a consequence of enhancement of signals converging on the GM-CSF or IL-2 gene, we measured the amount of endogenous GM-CSF and IL-2 proteins. Culture supernatants from Jurkat cells, transfected transiently with the active CN, were assayed for GM-CSF and IL-2 levels, following treatment of various reagents. Transfected cells with the control vector pBJ5 produced GM-CSF and IL-2 only when stimulated with PMA/A23187 (Figure 5, A and B). In contrast, transfection with pBJ5-CNΔA revealed that expression of the constitutively active CN synergized with PMA to induce production of GM-CSF and IL-2, both of which were inhibited by the addition of CsA (Figure 5, A and B). The levels of GM-CSF and IL-2 production were, however, lower compared with those induced by PMA/A23187 in the pBJ5-transfected cells. Therefore, behavior of the plasmids pKC17 and pIL-2 Luc-1 reflects the levels of expression of the endogenous GM-CSF and IL-2 genes, respectively.

Exogenously Expressed CN Augments CLE0-Dependent Transcription

To characterize CN responsive element(s) in detail, we divided the region between −91 and −37 into two segments, followed by insertion into the minimal GM-CSF promoter (position −36 to +27). p(GM-κB/GC-box)$_2$ CAT and p(CLE0)$_2$ CAT contain two copies of segment −91 to −72 and segment −70 to −37, respectively (Figure 6B). PMA/A23187 treatment induced transcription from p(GM-κB/GC-box)$_2$ CAT in a lower level than that from p(CLE0)$_2$ CAT. Co-transfection of p(GM-κB/GC-box)$_2$ CAT with pBJ5-CNA and pBJ5-CNB stimulated transcription 1.5-fold compared with pBJ5, whereas co-transfection of p(CLE0)$_2$ CAT with pBJ5-CNA and pBJ5-CNB enhanced transcription twofold compared with pBJ5 (Figure 6A). The level of stimulation by CN in p(CLE0)$_2$ CAT was more than threefold

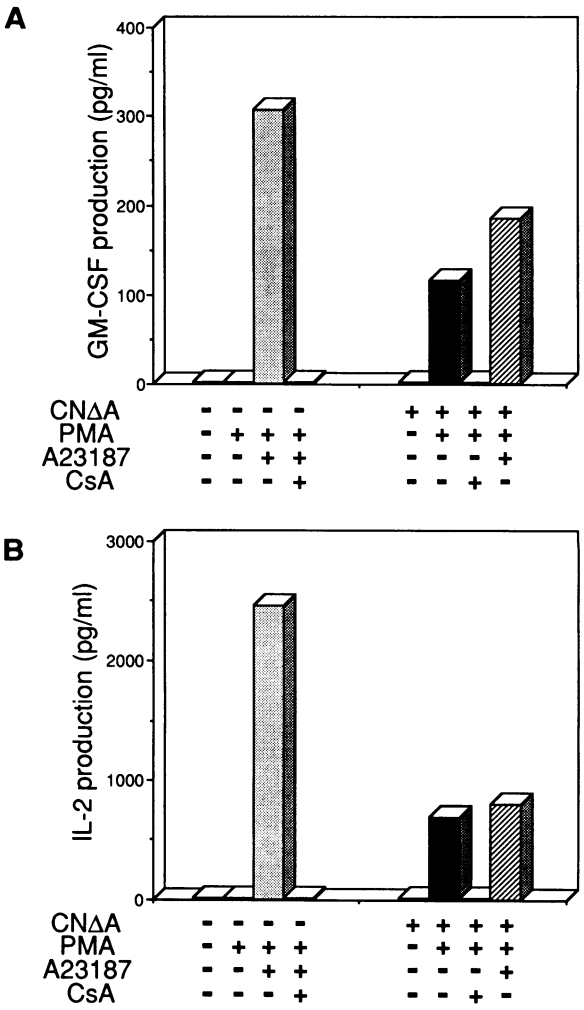

Figure 5. Constitutively active CN acts in synergy with PMA to induce production of endogenous GM-CSF and IL-2 in Jurkat cells. Cells (4×10^7) were transfected by electroporation either with 100 μg of pBJ5 or pBJ5-CNΔA. After 36 h incubation, the cells were harvested, suspended in culture medium, divided into 4 wells of 16-well plates and stimulated for 24 h with PMA (50 ng/ml) and/or A23187 (1 μM) in the presence of CsA (0 or 1.0 μg/ml). Results presented are the averages of 3 independent experiments. Detection limits in ELISA were 40 pg/ml for GM-CSF and 200 pg/ml for IL-2.

higher than that in p(GM-κB/GC-box)$_2$ CAT. It may well be that the CLE0 element more than the GM-κB/GC-box sequence is responsible for both the inducibility by PMA/A23187 and the potentiation by CN. We then constructed a plasmid containing three copies of the CLE0 element (position −57 to −37) upstream of the basal GM-CSF promoter (Figure 6D). Co-transfection of p(CLE0)$_3$ CAT with pBJ5-CNA and pBJ5-CNB showed that expression of CN augmented the CLE0-dependent transcription threefold compared with pBJ5 (Figure 6C). These results indicate that like the NF-AT site of the IL-2 gene, the CLE0 element of the GM-CSF

gene is one target for enhancement of transcription by CN and for induction through the PKC- and Ca^{2+}-signaling pathways in activated T cells (Figure 7).

DISCUSSION

In T cells, co-stimulation with phorbol esters (PMA) and Ca^{2+} ionophores (A23187) leads to considerable activation of several lymphokine genes. We have found that production of IL-2 or GM-CSF shows strong synergy between PKC- and Ca^{2+}-signaling pathways both in murine cloned T cells (Arai *et al.*, 1992) and in Jurkat cells (Figure 1A). In cloned T cells, certain lymphokines are partially activated by PMA alone (IL-5, IL-6, and IL-10) or A23187 alone (IL-3, IL-4 and interferon [IFN]-γ) (Arai *et al.*, 1992). CsA completely inhibits the IL-2 and GM-CSF production induced by PMA/A23187 in both these T cell lines (Figure 1B). In the cloned T cells, CsA almost completely blocks the IL-3, IL-4, and IFN-γ production induced by PMA/A23187 (Arai *et al.*, 1992). Our results are consistent with the observation that in activated human lymphocytes, CsA and FK506 inhibit accumulation of transcripts from early T cell activation genes, including IL-2, IL-3, IL-4, IFN-γ, TNF-α, and GM-CSF (Tocci *et al.*, 1989).

Earlier studies on the IL-2 gene showed that both transcriptional activation through two NF-AT sites and an Oct-1 (octamer factor-1)/OAP-40 (octamer-associated protein-40) site (Figure 7) and the respective DNA bindings are highly sensitive to CsA and FK506 (Riegel *et al.*, 1992). NF-AT consists of two factors: a ubiquitous nuclear factor ($NF\text{-}AT_n$), identified as AP-1 consisting of Jun-Fos heterodimers such as Fra1/JunB (Jain *et al.*, 1992; Boise *et al.*, 1993), and a T cell-specific, pre-existing component ($NF\text{-}AT_c$), whose translocation from cytoplasm into nucleus is inhibited by CsA and FK506 (McCaffrey *et al.*, 1993; Northrop *et al.*, 1993). In foregoing work (Masuda *et al.*, 1993), we demonstrated that the CLE0 element of the GM-CSF promoter is recognized by NF-CLE0 γ with a relatively weak affinity. NF-CLE0 γ is induced by PMA/A23187, abolished by the addition of CsA (Figure 2B) and competitively inhibited by NF-AT oligonucleotides (Figure 2C). NF-CLE0 γ has other characteristics similar to NF-AT, since it appears to be composed of AP-1 and 120 kDa protein (Tokumitsu *et al.*, 1993). The CLE0 element shares partial homology with the NF-AT-binding site (TTTCCTC, Figure 7), which is closely related to the consensus binding sequence for Ets-family proteins (Wasylyk *et al.*, 1993). Interestingly, the CLE0 sequence contains an AP-1-like site juxtaposed directly with the Ets-binding site (Figure 7). The CLE0-AP-1 site (TTAATCA) is similar to the AP-1 consensus sequence (TGAG/CTCA), and differences are probably not critical for binding of the Jun-Fos heterodimer (Risse *et al.*, 1989) since AP-1 can bind to the CLE0 sequence with a high affinity (Tokumitsu *et al.*, 1993). NF-CLE0 α and β are induced mainly by PMA (Figure 2B) and share most properties with AP-1 (Masuda *et al.*, 1993).

CsA and FK506 tightly bind distinct families of immunophilin (Schreiber and Crabtree, 1992; Sigal and Dumont, 1992), and the resulting complexes associate with CN to inhibit phosphatase activity (Fruman *et al.*, 1992). It has been reported that CN is expressed at a low level in T cells (Kincaid *et al.*, 1987, 1990), whereas the immunophilins are relatively abundant (Sigal and Dumont, 1992). We showed that as in the case of the IL-2 gene (Figure 3B), overexpression of CNA/CNB augments transcription of the GM-CSF gene and restores the transcription inhibited by CsA in Jurkat cells (Figure 3A). We also found that the constitutively active form of CNA subunit synergizes with PMA to stimulate transcription from the GM-CSF promoter (Figure 4A), as described in the earlier study on the IL-2 promoter (O'Keefe *et al.*, 1992). Finally we demonstrated that the constitutively active CN acts in synergy with PMA to induce production of endogenous GM-CSF and IL-2 (Figure 5, A and B). In both cases, the levels of induction by PMA/CNΔA are lower than those induced by PMA/A23187 in mock-transfected cells, as probably related to transfection efficiency. This would not, however, account for the low levels of lymphokine production induced by PMA/A23187 in CNΔA-transfected cells. Furthermore, CLE0 sequence-dependent transcription is stimulated by the expression of CNA/CNB (Figure 6C) as well as by that of the constitutively active type. Unexpectedly, the transcriptional enhancement of $p(CLE0)_2$ CAT and $p(CLE0)_3$ CAT constructs by CN was not inhibited by CsA. Transcription from tandemly repeated AP-1 sequences was seen to be stimulated by FK506 in the presence of PMA/ionomycin (Ullman *et al.*, 1993). This may account for the failure of CsA in repression of the transcriptional enhancement by CN, since the effect of the strong AP-1-binding site in the CLE0 sequence may become more dominant than that of the weak NF-AT-like-binding site when the CLE0 element is multimerized.

There has been no direct evidence that endogenous CN is implicated in activation of the GM-CSF and IL-2 genes through Ca^{2+} signaling. Co-transfection experiments can be done to determine whether overexpression of the auto-inhibitory domain of the CNA subunit reduces activation of these promoters in response to PMA/A23187, since a high dose of a peptide to this domain inhibits the phosphatase activity in vitro (Hashimoto *et al.*, 1990). A dominant negative mutant of CNA subunit, which lacks only the phosphatase activity, may also be useful to block activation of endogenous CN in response to the Ca^{2+} signaling by competition. These strategies, however, may not be feasible in transient transfection assays because at least two subtypes of the CN catalytic subunit have been identified by molecular cloning (Kincaid *et al.*, 1990, 1991).

It also has been reported that in addition to NF-AT and OCT-1/OAP-40, CsA and FK506 inhibit, by ~50%, both NF-κB binding to the immunoglobulin κ light chain enhancer (Ig-κB) sequence and the ability of multimerized Ig-κB sequences to activate expression from a reporter plasmid in response to PMA/ionomycin (Emmel *et al.*, 1989; Mattila *et al.*, 1990). NF-κB binding is less sensitive to these immunosuppressants since it is largely induced by PMA alone. The increased level of NF-κB activity (binding and transcription) induced by addition of ionomycin is completely inhibited by CsA and FK506 in Jurkat cells (Emmel *et al.*, 1989; Mattila *et al.*, 1990). We reported earlier that authentic NF-κB binding to the GM-κB site is weaker than that to the Ig-κB sequence (Tsuboi *et al.*, 1991). Since PMA/A23187-induced binding to the GM-κB site is inhibited down to a PMA level by CsA (Figure 2A), we cannot exclude the possibility that the GM-κB site may be another target for CsA effects; this contribution, if any, would be less significant than that of the CLE0 element. There are reports that the Ig-κB and HIV-κB sequences can be recognized by an NF-AT-like, CsA-sensitive factor under certain conditions of binding reaction or stimulation (Schmidt *et al.*, 1990; McCaffrey *et al.*, 1992). These κB sequences contain GGGACTTTCC and share homology with the above Ets-binding site (TTTCC). We have already purified NF-AT from nuclear extracts of PMA/A23187-stimulated Jurkat cells (Tokumitsu *et al.*, 1993); purified NF-AT fails to bind to the GM-κB sequence of the GM-CSF promoter. However, certain κB sequences may be other candidates for the CsA action through NF-AT-like proteins.

The CLE0 element of the GM-CSF promoter seems to be one of the targets for the CsA and CN actions (Figure 7), as described elsewhere (Randak *et al.*, 1990). Mobility shift assays (Figure 2A) and co-transfection experiments (Figure 6A) suggested that the GM-κB sequence may be another target for the CsA and CN effects. Transfection experiments using GM-CSF CAT constructs with the CLE0 or GM-κB/GC-box mutation revealed that cooperation between the CLE0 and GM-κB/GC-box elements is required for both maximal activation of the GM-CSF promoter and its enhancement by exogenous CN expression. It has been reported that an intergenic enhancer, located between human GM-CSF and IL-3 genomic genes, may participate in the inducibility of both genes and be a target for CsA inhibition. (Cockerill *et al.*, 1993). This enhancer contains four AP-1 binding sites, three of which are associated with an NF-AT-like factor and are similar to the CLE0 element (Cockerill *et al.*, 1993). To account for activation of the endogenous GM-CSF gene as well as its sensitivity to CsA, interaction between the distal and proximal CLE0-like elements should be also considered in the mouse GM-CSF/IL-3 locus. We have pointed out that the CLE0 element of the GM-CSF gene shares ho-

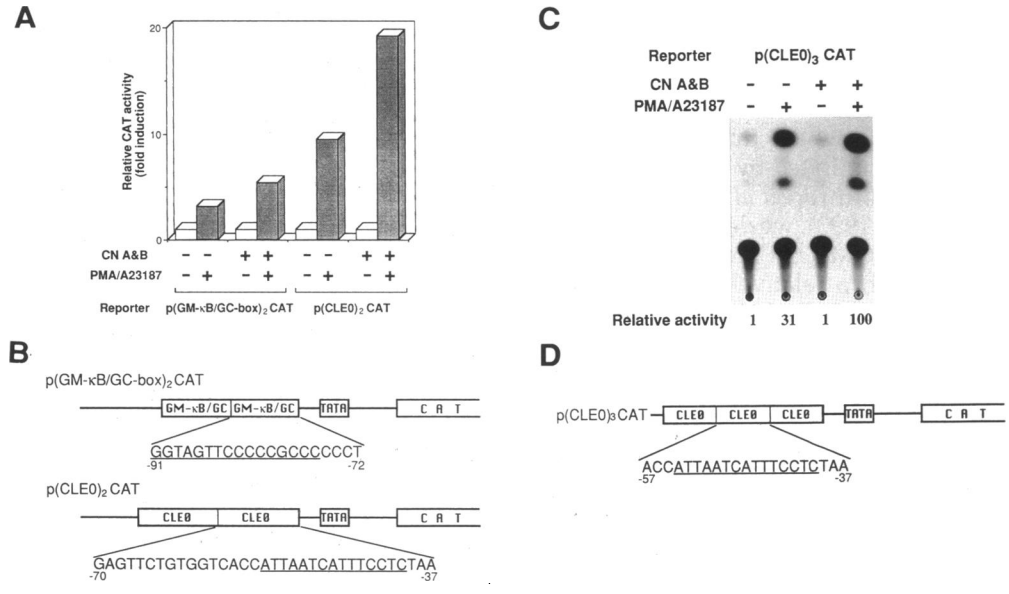

Figure 6. Exogenously expressed CN stimulates CLE0-dependent transcription in Jurkat cells. Jurkat cells (1×10^7) were co-transfected either with 5 μg of p(GM-κB/GC-box)$_2$ CAT (A), p(CLE0)$_2$ CAT (A) or p(CLE0)$_3$ CAT (C) along with either pBJ5 (20 μg) or pBJ5-CNA (10 μg) and pBJ5-CNB (10 μg), and divided into 2 aliquots. After 24 h incubation, the cells were stimulated for 15 h with PMA (50 ng/ml) and A23187 (1 μM). Results are shown as thin layer chromatogram (C), quantitated as fold induction of CAT activity compared with unstimulated cells (A and C), and are the averages of 3 independent experiments. Schematic representation of p(GM-κB/GC-box)$_2$ CAT (B), p(CLE0)$_2$ CAT (B), or p(CLE0)$_3$ CAT (D) carrying tandemly repeated GM-κB/GC-box or CLE0 sequence upstream of the minimal GM-CSF promoter (position −36 to +27). Underlines show the GM-κB/GC-box and CLE0 sequences essential for binding of respective factors. Means and SD are 1 ± 0.23, 3.1 ± 0.37, 1 ± 0.037, 5.4 ± 0.27, 1 ± 0.059, 9.5 ± 1.2, 1 ± 0.015, and 19 ± 0.81 (A); and 1 ± 0.074, 31 ± 3.0, 1 ± 0.018, and 100 ± 3.0 (C).

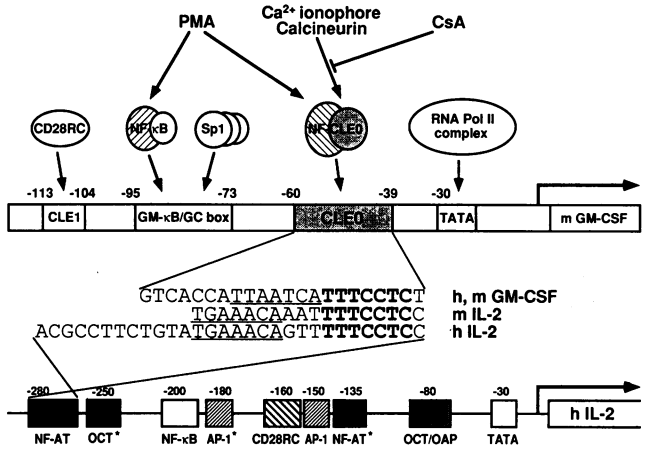

Figure 7. A model for coordinated regulation of the GM-CSF and IL-2 genes in activated T cells through PMA/Ca^{2+} inophore-responsive and CsA-sensitive elements, associated with NF-AT or NF-AT-like factor. NF-AT-binding sequences in both human and mouse are shown as the reverse strand. Bold letters in the DNA sequences indicate Ets-binding sites, and underlines show AP-1-like sites. Asterisks in the Il-2 promoter depict a low-affinity binding site of the respective factor. Binding sites for CD28-responsive complex (CD28RC) are described elsewhere (Fraser and Weiss, 1992).

mology with the regulatory regions of the IL-4 and IL-5 genes in both human and mouse (Miyatake *et al.*, 1991). We also found a homologous sequence with the CLE0 element in the 5'-flanking region of the mouse IL-3 gene (AGAGCCAGGCTACTTCCTCC) between positions −262 and −243 (Miyatake *et al.*, 1985). CsA blocks production of GM-CSF, IL-2, IL-3, IL-4, and IL-5, induced by PMA/A23187 in the murine cloned T cells as described above (Arai *et al.*, 1992). Therefore, we assume that the CLE0-like sequence may be involved in coordinated induction of the GM-CSF, IL-2, IL-3, IL-4, and IL-5 genes through the Ca^{2+}- and PKC-signaling pathways mediated during T cell activation. Use of mouse type 2 helper T cell clones, which produce IL-3, IL-4, IL-5, and GM-CSF will facilitate testing as to whether CN or CsA affects transcription of these genes through the CLE0-like sequence.

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