Positive and Negative Regulation of the Composite Octamer Motif of the Interleukin 2 Enhancer by AP-1, OCT-2, and Retinoic Acid Receptor

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Summary

The differentiating agent retinoic acid (RA) has been previously reported to interfere with 12-O-tetradecanoyl-phorbol-13-acetate (TPA)/Ca^{2+}-induced signals for the regulation of the −96 to −66-bp octamer motif found in the enhancer for the interleukin (IL)-2 gene, which encodes a major T lymphocyte growth factor. The IL-2 octamer motif is a composite *cis*-element which binds Oct-1 and Oct-2 as well as a TPA/Ca^{2+}-inducible nuclear factor, previously termed octamer-associated protein (OAP^{40}). We show here that Oct-2, despite the presence of an active transcriptional activation domain, requires TPA/Ca^{2+}-induced signals to strongly transactivate the IL-2 octamer motif in Jurkat T cells. This Oct-2–dependent transactivation is inhibited by RA. The presence of an intact COOH-terminal domain of Oct-2 contributes to both TPA/Ca^{2+}-induced transactivation and the RA-mediated repression. We also show that both *Fos* and *Jun* components of the AP-1 factors participate in the OAP^{40} complex. Furthermore, transfected *c-jun*, *jun*-B, *jun*-D, *c-fos*, or *Fos*-B expression vectors partially substitute for TPA and Ca^{2+} and cooperate with Oct-2 for the transactivation of the combined OAP/octamer *cis*-element. Mutations of the genuine octamer-binding site abrogate both the binding of Oct-1 and Oct-2 and the TPA/Ca^{2+}-induced transactivation of the OAP/octamer motif. OAP confers to Oct-2 responsivity to both TPA/Ca^{2+} and RA, since specific mutations of the AP-1/OAP–binding site significantly reduce the transactivation by Oct-2 in response to TPA and Ca^{2+} and abolish the inhibition by RA. Furthermore, retinoic acid receptor (RAR) α is able to inhibit in vitro the formation of the complex between the nuclear AP-1/OAP and its specific binding site, resulting in the interference with Oct-2–dependent *cis*-regulatory function of this AP-1 element. Therefore, we propose that the TPA/calcium-activated AP-1/OAP element is the main target of positive or negative regulatory signals influencing the IL-2 octamer motif, through synergism with Oct-2 and antagonism by RAR.

Retinoic acid (RA)[1] exerts a wide variety of effects on embryonic development, cell differentiation, and growth through regulation of growth factors and homeotic genes and protooncogenes (1–7). Retinoids bind to several receptors (e.g., retinoic acid receptors [RAR] α, β, and γ and retinoid X receptor [RXR] α, β, and γ and their isoforms), which combine with each other to form a variety of dimers able to bind and transactivate specific *cis*-elements of target genes (for review see reference 8). The range of genes controlled by retinoids is further broadened by the ability of RAR and RXR to control the expression of genes encoding other transacting factors, such as the homeotic *Hox* genes, nuclear protooncogenes, and the development-related octamer-binding proteins (1, 4, 6, 9–12). This latter class of proteins belongs to the POU family of transacting factors characterized by a homeodomain DNA-binding region (13, 14). Transcrip-

[1] *Abbreviations used in this paper:* CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; GR, glucocorticoid receptor; NFAT, nuclear factor of activated T cells; PKC, protein kinase C; RA, retinoic acid; RAR, retinoic acid receptor; RARE-β, RAR-responsive element of the RARβ promoter; RRL, rabbit reticulocyte lysate; RXR, retinoid X receptor; tk, thymidine kinase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

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tional regulation by RAR and other nuclear receptors also occurs through antagonism or synergism with distinct families of transacting factors, including AP-1 (15–21; for review see reference 22).

The influence of RAR on lymphoid T cell differentiation and growth is suggested by the ability of RA to modulate the expression of the human and murine IL-2 genes, which encode a major T lymphocyte growth factor (23, 24) involved in the control of T cell development (25, 26). The use of retinoids as therapeutic agents and the subsequent potential immunosuppression and influence on development underline the importance of elucidating their regulatory role in the IL-2 gene. The IL-2 promoter contains several control elements, which mainly require a variety of transacting factors activated by protein kinase C (PKC) and calcium-mediated signals (for reviews see references 27 and 28). We have previously reported that one target of the RAR-mediated inhibition of the PKC- and calcium-mediated activation of the IL-2 enhancer is an octamer motif at a position −96 to −66 bp upstream of the start site (23). This motif binds the inducible nuclear factor(s) OAP[40], whose composition includes jun protein (29, 30), as well as both Oct-1– and Oct-2–transacting factors (29, 31, 32), which are, therefore, potential targets of RA-mediated regulation. Whereas Oct-1 is ubiquitous, Oct-2 has a more restricted pattern of cell distribution, being expressed in B cells and in some T cells (31, 32). Furthermore, both transacting factors are regulated by stimuli that activate the IL-2 gene (29, 31, 32). Specifically, Oct-2 levels are upregulated by antigen stimulation in some T cells (32).

The presence of distinct Oct factors regulating cytokine gene expression suggests that they might be responsive to differential regulatory pathways. Indeed, distinct transcriptional requirements for Oct-1 and Oct-2 have been reported. Whereas Oct-1 is competent in enhancing transcription from snRNA promoters, it requires supplementation by auxiliary factors, which provide transcriptional activation domains (e.g., the herpes virus VP16 protein harbors a strong acidic transactivation domain) to activate mRNA promoters (13, 33). In contrast, Oct-2 harbors a strong COOH-terminal transcriptional activation domain for mRNA promoters, which when replacing the Oct-1 COOH terminus, can provide transcriptional activation (13, 34). The OAP[40] complex may represent auxiliary factors required for Oct-1 function. However, the requirements of Oct-2 for the regulation of the IL-2 promoter remain to be elucidated.

In this study we have addressed the molecular events implicated in the Oct-2–dependent activation and RA-induced downregulation of the octamer motif of the IL-2 enhancer. We show that the IL-2 octamer motif is a composite binding site for Oct-2 and a *fos*- and *jun*-containing AP-1 complex, whose functional cooperation is required to fully enhance transcription. RARα is able to inhibit the formation of the AP-1-DNA complex in vitro, resulting in an interference with the Oct-2–dependent *cis*-regulatory function of this AP-1 element. Therefore, the AP-1 element appears to be the main target for positive or negative regulatory signals that influence the IL-2 octamer motif, either through cooperation with Oct-2 or antagonism by RAR.

Materials and Methods

Plasmid Constructs. The plasmid pIL2CAT contains the IL-2–flanking region from −575 to +47 bp driving the chloramphenicol acetyltransferase (CAT) gene (35). All reporter constructions based on the pBLCAT2 vector were prepared by use of a vector in which the AP-1 site in the plasmid backbone had been deleted, as previously described (17). IL-2-OAP/OCT-tk-CAT was constructed by inserting four copies of the proximal (−96 to −66-bp) IL-2 octamer motif into either the pBLCAT2 vector (36) (including the octamerless [−105-bp] thymidine kinase [tk] promoter driving the CAT gene) or the (−37-bp) tk-CAT vector (37), as previously described (23). All of the plasmids containing mutated (m) or deleted (Δ) sequences of the IL-2 octamer motif (described in Fig. 6) were prepared in the same way. Wild-type deletion mutants and chimeric forms of Oct-1 and Oct-2 cDNAs, cloned in pCG expression vector containing the human CMV promoter, were provided by Dr. W. Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (34). pRcRSV-OCT2 was constructed by inserting the Oct-2 cDNA in the pRcRSV vector (Invitrogen, San Diego, CA). Expression vectors encoding hRARα and its mutant forms (Δ1–81 and Δ81–152) and RXRα were provided by Dr. P. Chambon (Laboratoire de Génétique Moléculaire des Eucaryotes du CNRs, Strasbourg, France) and Dr. R. Evans (Howard Hughes Medical Institute) (19, 38). The plasmid tk-TREp2-CAT contains the RA-responsive element driving the CAT gene through the tk promoter (39). pAP-1coll-tk-CAT contains five copies of the collagenase AP-1 site in front of the tk promoter (40). JMneo (c-*fos* expression vector), *fos*-Bneo (*fos*-B expression vector), their control vector pRΔXneo, and expression vectors for c-*jun* and *jun*-B under the control of the Rous sarcoma virus (RSV) promoter (RSV c-*jun* and RSV *jun*-B) and their control vector pUC-RSV were as previously described (41–44). The *jun*-D expression vector, containing *jun*-D cDNA under the control of the CMV promoter in the pCMV-1 vector, has been previously described (17).

Cell Culture and DNA Transfection. The human Jurkat T cell lines (T-77 clone and a subclone partially resistant to RA) were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics (50 IU/ml penicillin and 50 μg/ml streptomycin; Flow Laboratories, Ayrshire, Scotland). HeLa cells were grown in DMEM (Flow Laboratories) supplemented with 10% FCS and antibiotics as described above. Jurkat cells were treated with 30 ng/ml 12-O-tetradecanoyl-phorbol-13-acetate (TPA), 1 μg/ml A23187 or 2 μg/ml ionomycin (Sigma Chemical Co., St. Louis, MO), in the presence or absence of 1 μM RA (Sigma Chemical Co.).

Transfection of Jurkat cells were carried out either by the DEAE dextran method as previously described (23, 45) or by electroporation. In the latter case, plasmid DNA mixtures were added to 10^7 Jurkat cells/ml medium without serum and antibiotics and electroporated in a gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA) using 350 V and 960 μF capacitance, and then further cultured in complete growth medium. All cells were cotransfected with plasmid pCH110 (Pharmacia, Uppsala, Sweden) as an internal control for transfection efficiency. 24 h after transfection, cells were treated with the drugs indicated above. After a further 24 h, cells were harvested and protein extracts were prepared for the CAT and β-galactosidase assays, as previously described (23). Data were quantified by autoradiography and liquid scintillation counting and normalized to the levels of β-galactosidase expression of cotrans-

fected pCH110 vector obtained from the same cell extract. The levels of transfected Oct proteins were monitored by gel retardation assays and Western blotting (by use of anti-Oct-1 and -Oct-2 antibodies, described in reference 46).

Nuclear Extracts. Nuclear extracts from Jurkat T cells were prepared by a modification of a previously described technique (47). Cells were lysed by homogenization in 10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 10% glycerol, and 0.2% NP-40. Nuclei were centrifuged at 1,000 *g* and resuspended in two volumes of the above-described solution. KCl was added to a final concentration of 0.39 M, and nuclei were extracted at 4°C for 1 h and centrifuged at 100,000 *g* for 30 min. The supernatants were dialyzed against 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM PMSF, 1 mM DTT, and 20% glycerol, the debris removed by centrifugation, and stored at −80°C. Nuclear extracts from HeLa cells were prepared by homogenizing cells in 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, and 0.25 mM DTT. Nuclear fractions were extracted in 20 mM Hepes, pH 7.9, 20% glycerol, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.25 mM DTT, and 0.5 mM PMSF, dialyzed against 20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM PMSF, 1 mM DTT, and 20% glycerol, and cell debris were removed by centrifugation.

In Vitro Translation. RNA templates for in vitro translation reactions of hRARα, hRXRα, Oct-1, and Oct-2 were generated from plasmids (pGEM3) containing the cDNAs by SP6 polymerase (Promega Biotec, Madison, WI) and translated in vitro with rabbit reticulocyte lysate (RRL) according to the manufacturer's recommendation.

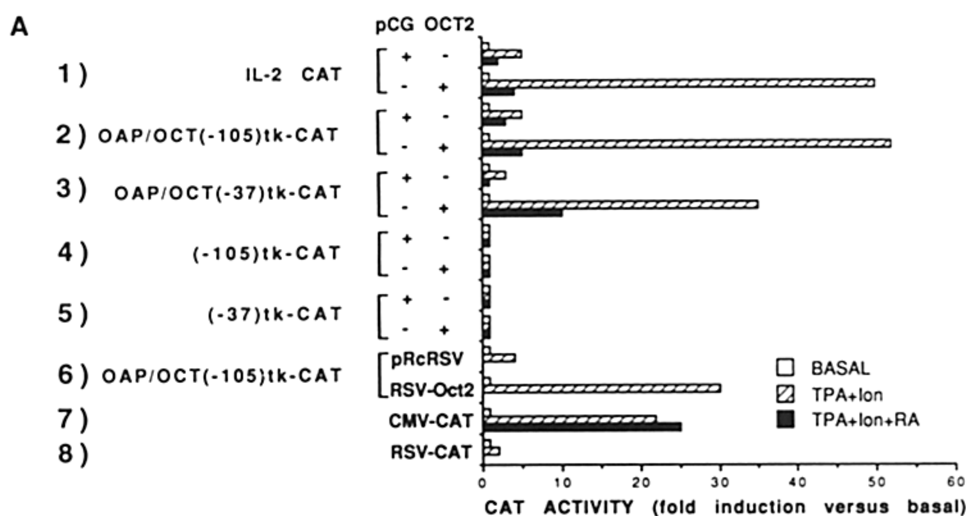
Bacterial Expression of RARα Protein. Full-length hRARα cDNA was inserted into pRSET B vector (Invitrogen) and expressed in *Escherichia coli* BL21(DE3)plysE. A single colony of *E. coli* BL21(DE3)plysE transformed with pRSET B-RARα was grown in Luria broth with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) at 37°C until cell density reached an OD_{600} of 0.7. Isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co.) was added to final concentration 0.4 mM for 3 h to induce hRARα protein expression. Recombinant protein was then purified on resin-charged columns (ProBond; Invitrogen) according to the manufacturer's recommendation. RARα protein was verified by Western immunoblot analysis using anti-RARα primary antibody Ab9α(hF) (provided by Dr. Chambon) diluted 1/1,500, as described (48).

Electrophoretic Mobility Shift Assay. ^{32}P-labeled probes (0.4 ng, 50,000 cpm) were incubated with 10 μg nuclear extracts of Jurkat cells for 20 min at room temperature in 15 mM Hepes, pH 7.9, 2.5 mM $MgCl_2$, 2 mM DTT, 40 mM KCl, 4% glycerol, and 2–5 μg poly(dl-dC) (Sigma Chemical Co.). Anti-pan-*fos* (provided by Dr. M. Iadarola, National Institutes of Health, Bethesda, MD) (49) or anti-pan-*jun* antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were incubated with nuclear extracts for 1.5 h at 4°C after binding reactions, described above. In other experiments, 5 μg Jurkat nuclear extracts and/or 5 μl in vitro synthesized RARα and RXRα or bacterially expressed RARα proteins were preincubated with 1 μg poly(dl-dC) in 10 mM Hepes, pH 7.9, 1 mM DTT, 2.5 mM $MgCl_2$, 10% glycerol, and a range of KCl concentrations of 10, 40, and 100 mM for 15 min on ice. After the addition of the RAR-responsive element of the RARβ promoter (RAREβ; 50) or OAP/OCT probe, the reaction mixtures were incubated for a further 25 min at room temperature. Anti-RARα antibody Ab9α(hF) was incubated with the mixture for 15 min at 4°C after the binding reaction. All binding reactions were performed in a final volume of 20 μl. Protein–DNA complexes were separated from free probe by gel electrophoresis on 4% polyacrylamide gels in 0.25× TBE buffer (0.23 M Tris-borate, 1 mM EDTA, pH 8.0) at 150 V for 1.5 h at room temperature. Gels were dried and exposed to film (XAR-5; Eastman-Kodak, Rochester, NY) at −80°C.

Results

TPA and Calcium Ionophore–mediated Induction of Oct-2–dependent Activation of the IL-2 Octamer Motif and Its Antagonism by RA. Activation of intact (−300 bp) IL-2 enhancer and the −96 to −66-bp IL-2 octamer motif fused to a heterologous tk promoter was demonstrated in Jurkat cells transfected with Oct-2 expression vector after treatment with TPA and ionomycin (Fig. 1). Transactivation of the IL-2 enhancer and IL-2 octamer motif driving either the −105-bp tk or the −37-bp tk promoters was significantly stronger in Jurkat cells transfected with Oct-2 expression vector after TPA and ionomycin treatment than that caused by endogenous Oct-1 in cells transfected with empty pCG vector (Fig. 1 *A*). Accumulation of nuclear Oct-2–IL-2 octamer complex could be demonstrated in cells devoid of endogenous Oct-2 transfected with pCG-Oct2 vector, which encodes Oct-2 under the control of the CMV promoter (Fig. 1 *B*). The levels of this complex were augmented 10–20-fold by TPA and ionomycin treatment due to upregulation of the CMV promoter (Fig. 1, *A* and *B*). This drug-induced augmentation in Oct-2 levels alone would not appear to contribute to IL-2 octamer motif transactivation as CAT activity in the absence of TPA and ionomycin treatment was not significantly increased, despite similar levels of intracellular Oct-2 protein (Fig. 1, *B* and *C*; compare CAT activity and intracellular Oct-2 levels in cells transfected with 0.2 μg pCG-Oct-2 and treated with TPA/ionomycin to those transfected with 5 μg pCG-Oct-2 in the absence of TPA/ionomycin). Similar results were obtained when Oct-2 expression vector driven by the RSV promoter (RSV-Oct2) was transfected into Jurkat cells, which was not significantly influenced by TPA/ionomycin (51; Fig. 1 *A*). This would suggest a requirement for additional TPA/ionomycin-induced coregulatory signals in Oct-2–mediated transactivation of octamer motif. TPA/ionomycin-induced Oct-2–dependent transactivation of both the intact IL-2 and the −96 to −66-bp IL-2 octamer motif was significantly inhibited by RA (Fig. 1 *A*). As RA neither inhibited the activity of the CMV promoter, used to drive Oct-2 transcription (Fig. 1*A*), nor decreased the levels of Oct-2 protein resulting from transfection (Fig. 1 *D*), the inhibitory activity of RA likely resulted from interference with TPA/ionomycin-induced coregulatory signals. These data suggest that the −96 to −66-bp IL-2 octamer motif is the focal point for both RA-mediated inactivation and the TPA/ionomycin-mediated cofactor requirement of Oct-2–dependent IL-2 transactivation.

Delineation of TPA/Ionomycin and RAR-responsive Domains in Oct-2. To study the domains of the Oct-2 protein that are involved in TPA/ionomycin-induced transactivation and



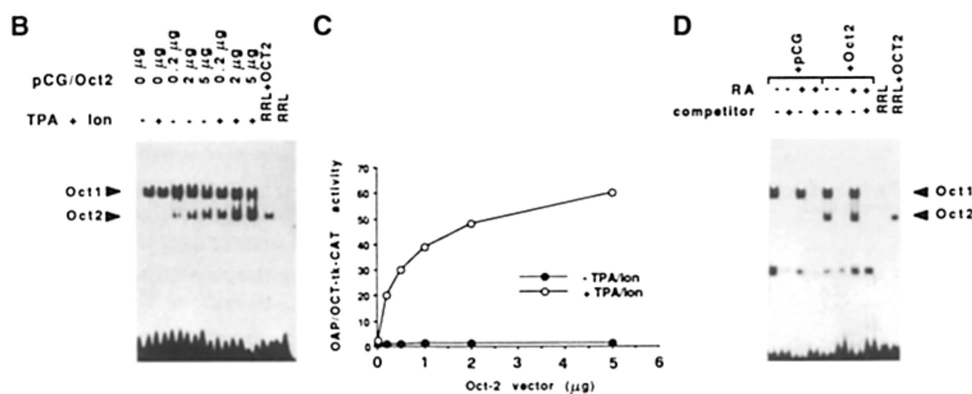


Figure 1. Oct-2 superinduces the IL-2 octamer motif. (*A*) Graphic representation of the fold induction of CAT activity compared with basal levels in CAT assays performed on cell lysates prepared from Jurkat cells cotransfected with IL-2-CAT (row 1), OAP/OCT(-105)-tk-CAT (row *2*), OAP/OCT(-37)-tk-CAT (row *3*), (-105)-tk-CAT (row *4*), or (-37)-tk-CAT (row *5*) cotransfected either with (+) or without (−) pCG vector (*pCG*) or pCG-Oct-2 expression vector (*Oct-2*). CAT activity was also assayed in cell lysates prepared from cells cotransfected with OAP/OCT (-105)-tk-CAT vector and either pRcRSV empty vector or Oct-2 encoding pRcRSV-vector (*RSV-Oct2*) (row *6*) or transfected with CMV-CAT alone (row *7*) or RSV-CAT alone (row *8*). All cells were transfected with 5 μg of the above-described CAT vectors plus 1 μg RARα expression vector. Extracts in all cases were prepared 24 h after treatment with medium alone (□), TPA and ionomycin (▨), or TPA and ionomycin in the presence of 1 μM RA(■). All treatments were performed 24 h after transfections. Results are expressed as mean (±SE from three to six experiments) fold induction of CAT activity observed in drug-treated versus -untreated cells (basal). Basal CAT activity values (expressed as pmol/h/mg protein) in the absence or in the presence of Oct-2, respectively, were as follows: 45 ± 8 and 55 ± 9 (in cells transfected with IL-2-CAT); 120 ± 15 and 190 ± 30 (in cells transfected with IL2OAP/OCT(-105)tk-CAT); 55 ± 7 and 80 ± 6 (in cells transfected with IL2OAP/OCT(-37)tk-CAT); 110 ± 10 and 105 ± 15 (in cells transfected with -105tk-CAT); and 60 ± 4 and 55 ± 5 (in cells transfected with −37tk-CAT). (*B*) Representative EMSA of Oct-2 octamer complexes in Jurkat cell nuclear extracts prepared after transfection with increasing amounts (0.2–5 μg) of pCG-Oct-2 expression vector as indicated and incubation for 4 h either in medium alone (−) or in the presence of TPA and ionomycin (+). Binding reactions contained either nuclear extracts, in vitro translated Oct-2 protein (*RRL+Oct2*), or unprogrammed RRL and ^{32}P-labeled ΔOAP/OCT oligonucleotide probe ATATGTAAAACATTT (see also Fig. 6). Arrows indicate specific Oct-1 and Oct-2 DNA complexes. Results shown are representative of two similar experiments. (*C*) Graphic representation of CAT activity assayed in cell extracts prepared from Jurkat cells, cotransfected with OAP/OCT(-105)-tk-CAT reporter and increasing amounts of pCG-Oct-2 vector, 24 h after treatment with (○) or without (●) TPA and ionomycin. (*D*) Representative EMSA showing the effect of RA on octamer complexes in Jurkat cell nuclear extracts prepared from cells transfected with pCG vector alone (*+pCG*) or with pCG-Oct-2 expression vector (*+Oct2*) after 4 h treatment with TPA and ionomycin in the absence (−) or presence (+) of 1 μM RA. Displacement of specific Oct complexes by a 50-fold excess of unlabeled competitor ΔOAP/OCT oligonucleotide (*competitor* +) is also shown. Last two lanes show binding reactions containing unprogrammed RRL or in vitro translated Oct-2 (*RRL+OCT2*), respectively. A ^{32}P-labeled ΔOAP/OCT oligonucleotide probe was used.

in the inhibitory activity of RA, we cotransfected several constructs encoding Oct-2 and Oct-1 proteins carrying deletions in the NH_2- or COOH-terminal regions or chimeric proteins together with the IL-2-OAP/OCT-(−105)tk-CAT reporter (Fig. 2). The levels of expression and integrity of these transfected wild-type mutant Oct-2 and Oct-2 proteins in each transfection were monitored by electrophoretic mobility shift assay (EMSA) of nuclear extracts from an aliquot of transfected cells. The complexes observed in EMSA were, in each case, consistent with expression of the full-length protein (Fig. 2 *B*). The analysis of the transfected Oct-2 and Oct-1 proteins by EMSA showed that all mutant or chimeric proteins accumulated within the cells to comparable levels under basal conditions and after TPA/ionomycin or RA treatments (Fig. 2 *B*). Oct protein levels analyzed by Western immunoblotting (with anti-Oct-2 and anti-Oct-1 antibodies; 46) corresponded to those determined by EMSA (data not shown). Similar TPA/ionomycin-induced transcriptional activation function and repression by RA was observed with Oct-2 proteins deleted of the proximal (94 amino acids, Oct-2ΔN1) or distal (adjacent glutamine-rich 62 amino acids, Oct-2ΔN2) NH_2-terminal regions compared with the wild-type protein (Fig. 2 *A*).

In contrast, deletion of the COOH terminus from residue 358 to the end (including a putative leucine zipper; Oct-2ΔC) decreased the transacting function (as evaluated by transfec-

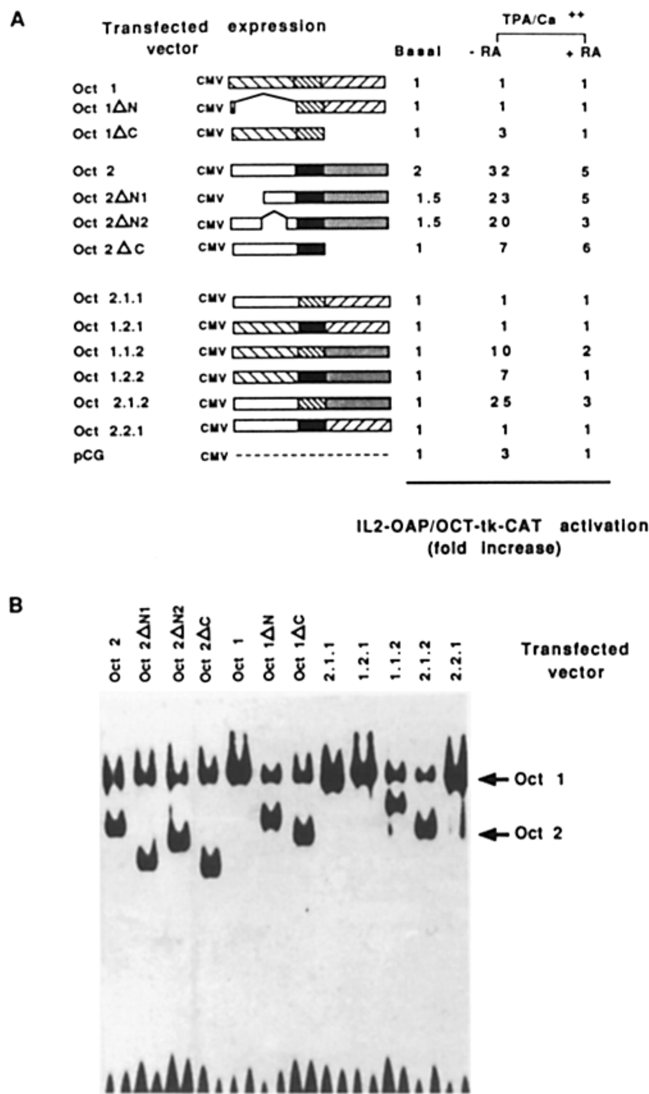


Figure 2. Differential transcriptional activation of the octamer motif by wild-type, mutant, and chimeric Oct-1 and Oct-2 proteins. (*A*) Jurkat cells were cotransfected with 5 μg IL-2-OAP/OCT(-105)tk-CAT, 1 μg RARα, and 1 μg of either the wild-type, mutant, or chimeric forms of the two Oct proteins, depicted on the left. Cells were treated with TPA and ionomycin and processed for CAT assay as described in Fig. 1. CAT activities relative to untreated (basal) cells transfected with pCG vector are represented. Basal levels in pCG-transfected cells were 100 ± 15 pmol/h/mg protein. CAT activities were normalized to the levels of transfected Oct proteins (considering the ratio between the protein levels and the CAT activity described in Fig. 1, *A* and *B*) and evaluated by EMSA of nuclear extracts from an aliquot of the cells. Results shown represent the average of three similar experiments (differing <20% from one another). (*B*) EMSA of the various wild-type and mutant Oct proteins observed in nuclear extracts from an aliquot of cells described in *A*. Assay and treatment conditions are as described in Fig. 1.

tion over a range of 0.5–5 μg plasmid) (Fig. 2 *A* and data not shown). Likewise, the antagonistic activity of RA on Oct-2ΔC was almost lost (Fig. 2 *A*). Surprisingly, transfection of the Oct-1 expression vector resulted in repression of the TPA/ionomycin-induced activation of the IL-2-OAP/OCT-tk-CAT construct. Whereas the Oct-2 COOH terminus was required for enhancer transactivation, the inhibitory activity of Oct-1 also mapped to the COOH terminus, according to data recently reported by Annweiler et al. (52) for the B cell–specific enhancer stimulation. In fact, transfection of Oct-1/Oct-2 chimeric constructs indicated that whenever the Oct-1 COOH terminus was present in the fusion protein (Oct-2.1.1, Oct-1.2.1, Oct-2.2.1), the transactivation of the octamer CAT was repressed (Fig. 2). Chimeric proteins containing the Oct-1 NH_2 terminus with Oct-2 POU domain and COOH terminus (Oct-1.2.2) or Oct-1 POU domain and Oct-2 COOH terminus (Oct-1.1.2) (transfected over a range of 0.5–5 μg plasmids) had comparably lower activity than Oct-2 NH_2 terminus-containing proteins (Fig. 2). They were, however, fully antagonized by RA. All of these data taken together show that an intact Oct-2 COOH-terminus contributes to both the TPA/ionomycin-mediated activation and RA-induced inhibition, suggesting that TPA/calcium and RA signal through an overlapping pathway. To investigate this pathway, we studied the nuclear factors binding to the octamer motif.

Characterization of the Composite IL-2 Octamer Motif: Presence of an AP-1 Complex and Transcriptional Activation by fos and jun. In addition to Oct-1 and Oct-2, a TPA/calcium-inducible nuclear factor, previously termed OAP^{40}, has been reported to bind to a region closely linked to the −96 to −66-bp IL-2 octamer–binding site (29). The OAP binding site shares five out of seven nucleotides with a canonical AP-1 binding motif (Fig. 3 *A*). The complex between the IL-2 octamer motif deleted from the Oct binding site (OAP/ΔOCT) and nuclear factors extracted from TPA/ionomycin-activated Jurkat T cells was displaced by competition with an excess of either unlabeled collagenase AP-1 (Fig. 3 *B*, *AP1 Coll*) or the proximal (−160 to −139-bp) AP-1 site of the IL-2 promoter (not shown), which has been shown to bind jun and fos proteins in activated T cells (21, 53). An unlabeled OAP/ΔOCT also competed for proteins from activated T cells that bind to an AP-1 site from the collagenase promoter (Fig. 3 *B*, *OAP*). Similar results were observed with HeLa cell nuclear extracts as a source of AP-1 factor (Fig. 3 *B*).

We also observed that antibodies against both fos and jun proteins were able to bind to the complex formed by nuclear factors (from both Jurkat and HeLa cells) and the OAP/ΔOCT sequence motif, whereas an unrelated antibody was uneffective (Fig. 3 *C* and data not shown). This result suggests that the protein complex that binds to the OAP/ΔOCT sequence contains *fos* and *jun* family members, which are components of AP-1. The OAP/ΔOCT sequence not only binds an AP-1 factor, but its *cis*-regulatory function is also activated by *jun* and *fos*. Fig. 4 shows that cotransfection of vectors encoding either c-*jun*, *jun*-B, or *jun*-D with pCG-Oct-2 (Oct2) in the absence of TPA/ionomycin treatment could partially substitute for PKC and calcium-mediated signals in the transactivation of the IL-2-OAP/OCT-tk-CAT reporter. Similar transactivation was observed by cotransfecting Oct-2 with c-*fos* or *Fos*-B, which are likely to form heterodimer complexes with the endogenous *jun*-D, which has been recently reported

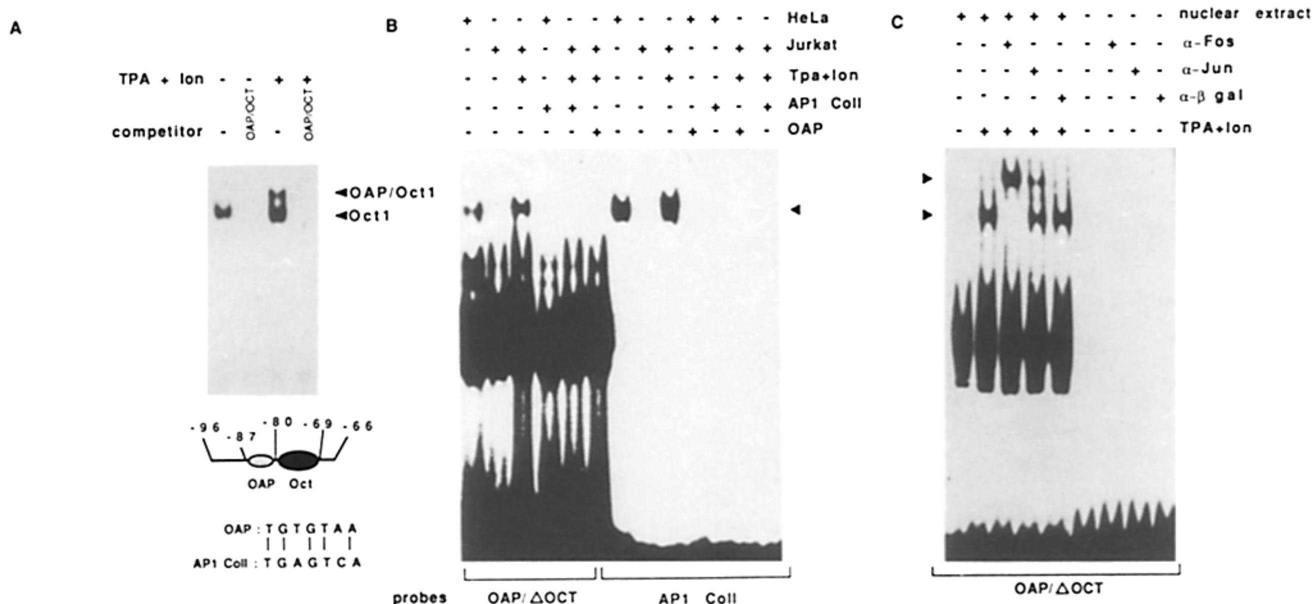


Figure 3. Detection of jun/fos/AP-1 factors binding to the IL-2 octamer motif in Jurkat cell nuclear extracts. (*A*) EMSA of nuclear extracts from Jurkat cells treated with or without TPA and ionomycin, binding to the OAP/OCT oligonucleotide represented on the bottom in the absence or presence of a 50-fold excess of unlabeled OAP/OCT competitor oligonucleotide. Arrowheads indicate the Oct-1 complex alone or the combined OAP/Oct-1 supershifted complex. (*B*) EMSA of unstimulated Jurkat and HeLa cell and 4-h TPA/calcium-stimulated Jurkat cell nuclear extracts binding to either the OAP site deleted of octamer sequences (OAP/ΔOCT, described in Fig. 6) or the AP-1 consensus sequence from the collagenase promoter (AP-1coll, 5′-GATCCGGCTGACTCATCA-3′). A 50-fold excess of unlabeled OAP or AP-1coll (*B*) competitor oligonucleotides were included in the binding reaction as indicated above each lane. Arrowheads indicate the specific TPA/Ca^{2+}-inducible AP-1 complex. (*C*) EMSA of nuclear extracts from either untreated or TPA/ionomycin-treated (for 4 h) Jurkat cells incubated with labeled OAP/ΔOCT probe for 30 min and then further incubated with antibodies against either fos (*α-Fos*), jun (*α-jun*) or β-galactosidase (*α-β gal*) proteins. Fos and jun antibodies recognize all members of the *fos* and *jun* families, respectively. Arrowheads indicate either the specific AP-1 complex or a complete or partial supershift of the TPA/ionomycin-inducible complex by *α-fos* and *α-jun*, respectively. Additional complexes moving faster than OAP band (shown in *B* and *C*) represent nonspecific complexes, as determined by competition using several competitor oligonucleotides (including nonspecific and self-competitors) as well as different amounts of Poly(dl-dC).

to be constitutively expressed in Jurkat cells (54). No significant increase in CAT activity was observed when *jun* and *fos* expression vectors were transfected in the absence of the Oct-2 vector (Fig. 4, pCG).

The OAP cis-Element Synergizes with the Oct-2 Octamer Complex and is Negatively Regulated by RAR. The ability of *fos* and *jun* to synergize with Oct-2 in enhancing the IL-2 octamer motif suggests that both elements may be required to drive transcription. To address this point, we introduced mutations or deletions in either or both OAP- and OCT-binding sites and tested their *cis*-regulatory activity on a heterologous tk promoter. We first studied the binding specificity of these sequences versus OAP, Oct-1, and Oct-2 proteins by testing their ability to compete for binding to radiolabeled OAP/OCT or OAP/ΔOCT probes. Oligonucleotides carrying substitution mutations or a deletion of the OAP binding site (mOAP/OCT and ΔOAP/OCT, respectively) competed, as well as the wild-type OAP/OCT motif for in vitro translated Oct-2 (Fig. 5 *A*, lanes *4–6*) or endogenous Oct-1 (Fig. 5 *B*, lanes *2, 4,* and *5*), but they did not bind the OAP factors (Fig. 5 *B*, lanes *4, 5, 14,* and *15*). Mutations or deletions of the octamer-binding sequence (OAP/mOCT and OAP/ΔOCT, respectively) abolished the DNA-binding ability of Oct-2 and Oct-1 (Fig. 5 *A*, lanes 7 and *8* and Fig. 5 *B*, lane *3*), but they still allowed binding of the OAP factors (Fig. 5 *B*, lanes *3, 12,* and *13*). An analysis of the functional activity of the mutated *cis*-regulatory motifs indicated that four copies of the double-mutated OAP/OCT element (m(OAP/OCT)-tk-CAT), OAP/mOCT (OAP/mOCT-tk-CAT), or OAP/ΔOCT (OAP/ΔOCT-tk-CAT) driving CAT expression through a tk promoter were unable to be transactivated by Oct-2 in response to TPA and ionomycin treatment (Fig. 6). This result suggests that the AP-1–binding OAP element has weak enhancer activity in the absence of concomitant Oct-2 binding. The abrogation of OAP binding (mOAP/OCT-tk-CAT and ΔOAP/OCT-tk-CAT) also impaired the ability of the octamer motif to be transactivated by Oct-2 in response to TPA and ionomycin by ∼90% (Fig. 6 *B*). This result suggests that Oct-2 has very weak enhancer activity in response to TPA and ionomycin without concomitant AP-1 binding. Thus, both *cis*-elements appear to require mutual cooperation for full enhancer properties. Of further interest is the observation that mutation or deletion of the OAP binding motif almost completely abrogated the ability to RA to antagonize the TPA/ionomycin-induced Oct-2–dependent activation of the IL-2 octamer binding site; this is probably due to the very low TPA/ionomycin-induced activation observed. Thus, RA inhibition was observed only when both

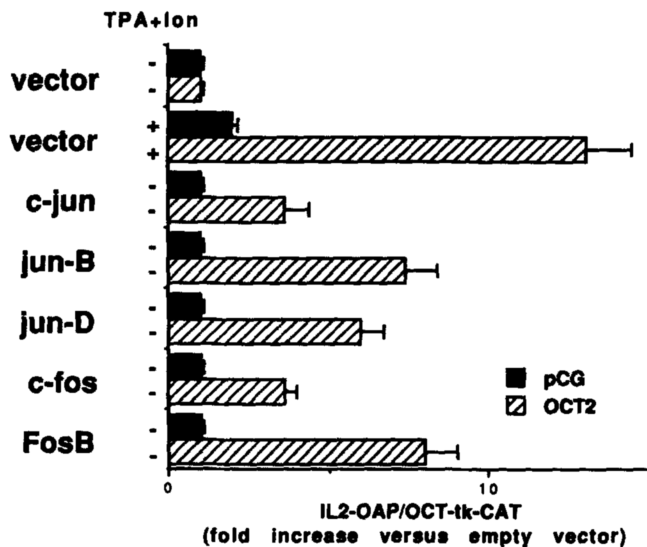


Figure 4. Transactivation of the IL-2-OAP/OCT *cis*-element by *jun* or *fos* and Oct-2 expression vectors in unactivated Jurkat cells. Empty vectors (pRΔXneo, pUC-RSV, or pCMV-1), *c-jun*, *jun*-B, *jun*-D, *c-fos*, or *Fos*-B expression vectors were cotransfected together with IL-2-OAP/OCT-tk-CAT and either pCG-Oct2 or pCG plasmids into Jurkat cells. Where indicated, cells were treated 24 h later with TPA and ionomycin (*TPA + Ion*). CAT assays in cell extracts were carried out 48 h after transfection. Results are expressed as the average (±SE from three experiments) fold increase of the CAT activity observed compared with untreated empty vector-transfected cells (basal activity 125 ± 20 and 180 ± 30 pmol/h/mg protein, in the absence and presence of Oct-2, respectively).

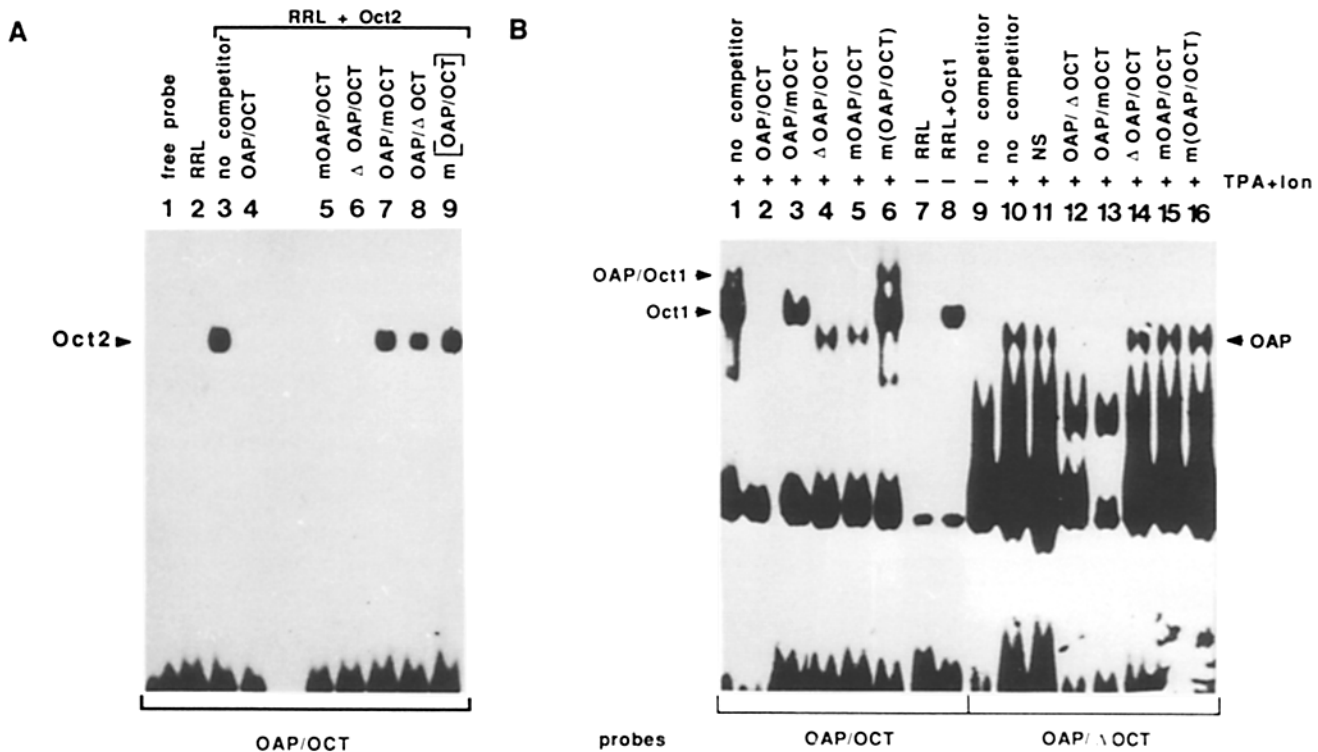


Figure 5. Binding specificity of OAP/OCT motif through competition assays using oligonucleotides carrying mutations or deletions in the OAP (*mOAP/OCT* and *ΔOAP/OCT*, respectively), octamer (*OAP/mOCT* and *OAP/ΔOCT*, respectively), or both sites [*m(OAP/OCT)*]. Mutated oligonucleotides are described in Fig. 6. (*A*) EMSA of in vitro synthesized Oct-2 protein (*RRL + Oct2, arrowhead*) or unprogrammed RRL binding to labeled OAP/OCT probe. The 50-fold excess of unlabeled competitor oligonucleotides are indicated above each lane. Arrowhead indicates the Oct-2 protein–DNA complex. (*B*) EMSA of nuclear extracts from Jurkat cells stimulated with TPA and ionomycin for 4 h, binding to either OAP/OCT or the OAP sequence alone (*OAP/ΔOCT*) as probes. For the competition assays, a 50-fold excess of the unlabeled oligonucleotides indicated above each lane (including a nonspecific sequence (*NS*)] were used. RRL and RRL+Oct1 indicate the complex formed by in vitro synthesized Oct-1 protein as described in Fig. 1. Arrowheads indicate the Oct-1 or OAP complexes alone or the combined OAP/Oct-1 supershifted complex.

A

	OAP	Octamer
IL2-OAP/OCT :	TTGAAAATATGTGTAA T	ATGTAAAACATTT
IL2-m(OAP/OCT) :	TTGAAAATATGTGTcc T	cgagg ACATTT
IL2-ΔOAP/OCT :	-------------------------A T	ATGTAAAACATTT
IL2-mOAP/OCT :	TTGAAAATATtTtTAA T	ATGTAAAACATTT
IL2-OAP/ΔOCT :	TTGAAAATATGTGTAA T	ATT----------------
IL2-OAP/mOCT :	TTGAAAATATGTGTAA T	ATaatActCA TTT

B

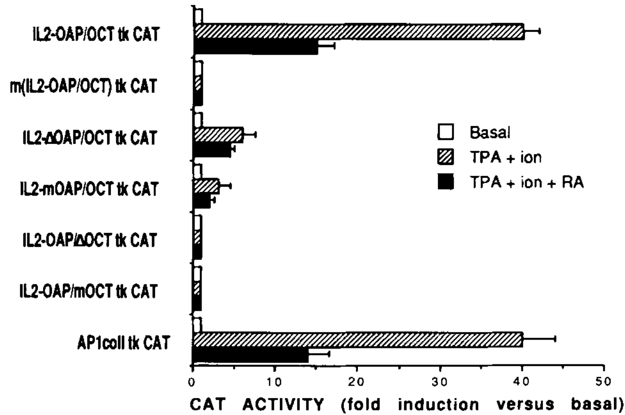


Figure 6. Functional analysis of Oct-2 transactivating activity in responsive sequences mutated either in the OAP (*IL-2-ΔOAP/OCT-tk-CAT, IL-2-mOAP/OCT-tk-CAT*), octamer (*IL-2-OAP/ΔOCTtk-CAT, IL-2-OAP/mOCT-tk-CAT*), or both sites (*m[IL-2-OAP/OCT]-tk-CAT*). (*A*) Description of the mutated oligonucleotides cloned as four-copy concatemers in front of the (-105)-tk-CAT fusion reporter. (*B*) Each reporter plasmid shown in *A* or AP1coll-tk-CAT (4 μg) was cotransfected into Jurkat cells together with pCG-Oct-2 and RARα (1μg) expression vectors. After 24 h, cells were treated with TPA and ionomycin or RA as indicated in Fig. 1 and further processed for CAT assay 24 h later. Results are expressed as the average (±SE from three experiments) fold increase of the CAT activity observed in drug-treated relative to untreated (*basal*) cells after subtraction of the CAT activity expressed in the absence of transfected Oct-2. Basal CAT activity values (*pmol/h/mg protein*) were 160 ± 25 (*IL2OAP/OCT-tk-CAT*); 100 ± 20 (*m[IL-2-OAP/OCT]tkCAT*); 138 ± 15 (*IL-2-ΔOAP/OCT-tk-CAT*), 127 ± 6 (*IL-2-mOAP/OCT-tk-CAT*), 94 ± 8 (*IL-2-OAP/ΔOCT-tk-CAT*), 80 ± 15 (*IL-2-OAP/mOCT-tk-CAT*), and 52 ± 5 (*APIcoll-tk-CAT*).

OAP- and Oct-2–binding sites were allowed to synergize with each other.

RAR has been shown to antagonize the collagenase AP-1 enhancer activity in HeLa cells (18, 19). Thus, a similar RAR-AP-1 antagonism might occur at the IL-2-OAP motif in T cells. We therefore studied the effect of RA on the TPA/ionomycin-induced activation of five copies of the collagenase AP-1 *cis*-element driving CAT transcription through the tk promoter. Fig. 6 *B* shows that RA treatment resulted in a decrease of the TPA/ionomycin-induced activation of AP-1-coll-tk-CAT cotransfected with an RARα expression vector into Jurkat cells.

RARα Inhibits OAP Binding Activity in Vitro. To study the RARα domains involved in the antagonism of the TPA/ionomycin-induced activation of OAP/OCT *cis*-elements, we examined the effects of RARα mutant expression vectors

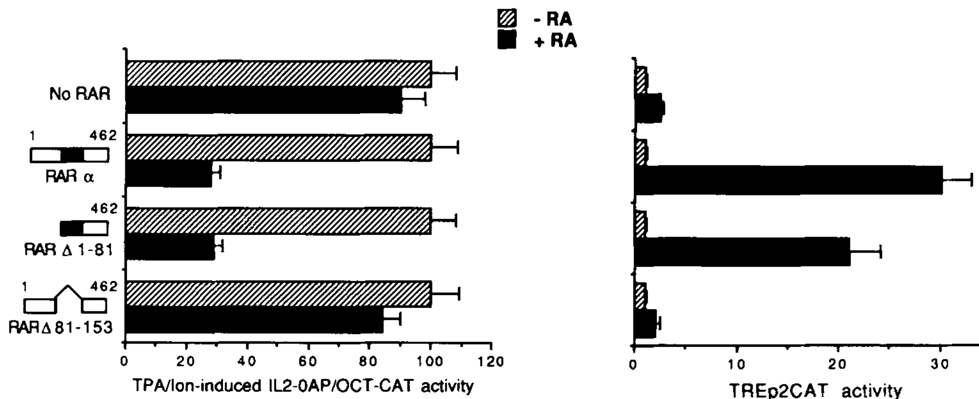


Figure 7. The RARα DNA-binding domain is necessary for the RA-mediated repression of the IL-2 octamer motif. Wild-type and deletion mutants (1 μg) of RARα receptor were cotransfected either with IL-2-OAP/OCT-tk-CAT (4 μg) or TREp2-CAT (4 μg) in an RA-resistant Jurkat cell subclone. On the left, the wild-type (RARα), amino (Δ1–81), and DNA-binding (Δ81–153) deleted receptors are schematically illustrated. Numbers over the various receptors indicate amino acid positions, and the filled box represents the DNA-binding domain. The cells were treated and processed for CAT assay as previously described. Results are expressed as CAT activity relative to the 100% TPA/ionomycin-induced activation of IL-2-OAP/OCT-tk-CAT in the absence of RA treatment. Similar levels of TPA/ionomycin-induced IL-2-OAP/OCT-tk-CAT activity in the absence of RA treatment were observed in untransfected cells and cells transfected with the various RAR expression vectors. For TREp2-CAT, results are expressed as RA-induced fold activation of CAT activity relative to RA-untreated cells. The various RAR expression vectors did not influence the TREp2-CAT activity in the absence of RA treatment. Results shown represent the average (±SE) of three experiments.

compared with the wild-type receptor on the activity of OAP/OCT-tk-CAT in the presence of CMV-Oct-2 cotransfected into an RA-resistant Jurkat subclone. Truncation of the NH_2 terminus of the receptor did not affect the inhibitory activity on OAP/OCT-tk-CAT expression and only slightly decreased the RA-induced activation of TREp2-CAT reporter, carrying an RA-responsive element driving CAT gene transcription (Fig. 7). In contrast, deletion of the receptor DNA-binding domain significantly reduced both RA-mediated repression of the TPA/ionomycin-induced activation of OAP/OCT-tk-CAT and enhancement of TREp2-CAT expression (Fig. 7). This suggests that direct DNA binding of RARα to the IL-2 sequences may be required for the effect on OAP/OCT *cis*-regulatory function. To address this point further, we tested the ability of in vitro synthesized, or bacterially expressed, recombinant RARα to bind to the −96 to −66-bp OAP/OCT IL-2 sequences in addition to the RAREβ. According to data previously reported (55, 56), RARα protein acquired RAREβ DNA-binding activity after complementation with in vitro synthesized RXRα: the presence of the receptor in the complex with RAREβ was confirmed by use of an anti-RAR antibody (Fig. 8 *C, left panel*), lanes *4*, *6*, and not shown). Interestingly, the complementation of RAR with nuclear extract from both untreated or TPA/ionomycin-activated Jurkat cells also conferred a distinct pattern of RAREβ DNA-binding activity, which was supershifted by the anti-RAR antibody (Fig. 8 *C, left panel*, lanes *9*, *11*, and not shown). This suggested that, in nuclear extracts, different cofactors were present that cooperate with receptor for DNA binding. In contrast, complementation with either RXRα or these different putative cofactors present in nuclear extract from both untreated or TPA/ionomycin-activated Jurkat cells was unable to confer the in vitro OAP/OCT motif DNA-binding ability to synthesized or bacterially expressed RARα proteins over a range of salt concentrations or DNA-binding incubation conditions (Fig. 8 *C, right panel*, lanes *5*, *6*, and not shown). The OAP/OCT motif was only capable of forming a complex

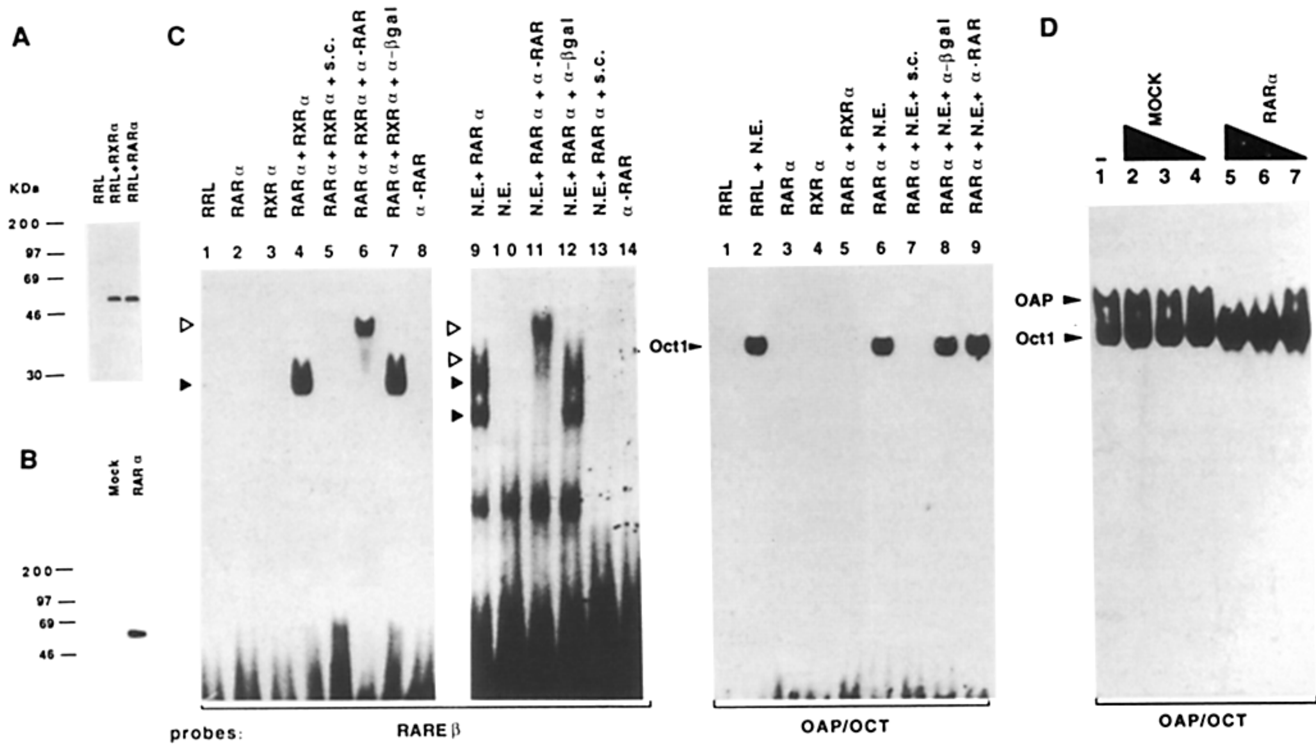


Figure 8. RARα does not bind the OAP/OCT motif but inhibits OAP-binding activity in vitro. (*A*) SDS-PAGE of [^{35}S]methionine-labeled RXRα and RARα translated by RRL (*RRL + RXRα or RRL + RARα*) or unprogrammed RRL. (*B*) Western immunoblotting of purified bacterially expressed RARα protein or the same amount of mock bacterial lysate. (*C*) DNA-binding ability (*solid arrowheads*) of in vitro synthesized RARα alone or complemented with either RXRα (lane *4* in *left panel* and lane *5* in *right panel*) or nuclear extract (*N.E.*; lane *9* in *left panel* and lane *6* in *right panel*) from untreated Jurkat cells. Binding reactions were carried out using either labeled RAREβ (5′-GATCCGGGTAGGGTTCACCGAAAGTTC-ACTCGA-3′) (*left panel*) or IL-2-OAP/OCT (*right panel*) oligonucleotides, in the absence or presence (*left panel*, lanes *5* and *13*; *right panel*, lane *7*) of a 50-fold excess of unlabeled probe as self-competitor (*s.c.*). Protein complexes with either labeled RAREβ or IL-2-OAP/OCT probes after addition of anti-RARα (*α-RAR*) (*left panel*, lanes *6* and *11*; *right panel*, lane *9*) or anti-β-galactosidase (*α-βgal*), (*left panel*, lanes *7* and *12*; *right panel*, lane *8*) antibodies to the binding reactions are also shown (*open arrowheads*). The binding of RXRα alone or unprogrammed RRL alone or in combination with nuclear extract is also represented. The specific complex between nuclear extract and the OAP/OCT probe is represented by Oct-1 (*arrow*). (*D*) RARα inhibits OAP binding in vitro. Nuclear extracts of TPA/ionomycin-stimulated Jurkat cells were incubated with labeled OAP/OCT probe in the absence (−) or presence of increasing amounts (1–6 μl) of bacterially expressed RARα (lanes *5–7*) or the same amounts of mock bacterial lysate (lanes *2–4*). Arrowheads indicate the Oct-1 complex alone or the combined OAP/Oct-1 (*OAP*) supershifted complex.

that included the Oct-1 factor present in nuclear extracts from either untreated or TPA/ionomycin activated cells, but not RAR, as confirmed by its insensitivity to anti-RAR antibody (Fig. 8 *C*, *right panel*, lane *9* and not shown). In contrast, the addition of recombinant RARα inhibited the amount of the supershifted complex formed by OAP and Oct-1 nuclear factors and the OAP/OCT DNA motif (Fig. 8 *D*, lanes *5–7*). The complex formed by nuclear Oct-1 alone and DNA was not affected by RARα (Fig. 8 *D*). Mock-transformed bacterial lysate did not affect the formation of either complex (Fig. 8 *D*, lanes *2–4*).

Discussion

Jun and fos Participate in the OAP Octamer Complex and Cooperate with Oct-2 for Transacting Function. We report here that Oct-2 is a very weak transactivator of the IL-2 octamer motif in the absence of phorbol ester and calcium ionophore, despite the presence of an active transcriptional activation domain. This would suggest a requirement for both PKC- and calcium-induced coregulatory signals in T cells. The requirement of coregulatory signals for Oct-2 transcriptional activity has also been recently reported in B cells. These signals, which are specific for B cells and absent in HeLa cells, appear to be accounted for by the presence of OCA-B factors, which interact with Oct-2 (57). Similar data have also been reported for E1A, which provides transcription activation function to Oct-4 via protein–protein interaction in embryonic stem cells (58). Thus, transactivation properties of Oct-2 and Oct-4 appear to result from the ability of specific cell types to provide cooperating factors that give them functional competence. In addition to these recently identified coactivators, we report here that another of the cell-specific factors that can cooperate with Oct-2 is the AP-1 complex in T cells. We suggest also that the presence of the AP-1 factor underlies the requirement for extracellular regulatory signals (PKC and calcium ionophore) in Oct-2–mediated transactivation in T cells. In this respect, we have further characterized the previously described OAP^{40} complex, which binds to a region closely linked to the octamer motif in the IL-2 promoter (29) as containing an AP-1 complex. Our data confirm the recently reported presence of *jun* in the OAP^{40} complex (30). In addition, we show the presence of immunoreactive fos protein(s) in the OAP^{40} complex. Furthermore, we show that overexpression of jun and fos proteins cooperate with Oct-2 for the transactivation of the IL-2 octamer motif, and that this cooperation is strictly required for full enhancer activity. The presence of an active transcriptional activation domain within Oct-2 suggests that the ability of AP-1 and other coregulatory factors to impart functional transcriptional competence to Oct-2 does not simply provide a transcriptional domain for a protein that lacks it, as in the case of VP16-Oct-1 interactions (13, 33).

We have mapped a part of the TPA/calcium-induced transacting function of Oct-2 to the COOH terminus, a region rich in heptad leucine repeats with the potential to form a "leucine zipper"-like structure. It remains to be elucidated whether this region is capable if interacting with leucine zipper regions in jun and fos proteins. We also observed differential transactivation of the OAP *cis*-element by distinct members of the *jun* and *fos* family, with *jun*-B and *Fos*-B being the most active. The composition of the AP-1 factors cooperating with distinct transacting factors, such as Oct-1 and Oct-2, may therefore by influential in determining the transacting function of the final complex. This may explain the different transactivating function of *jun*-B reported in this study compared with that described previously (30). Such a selection for AP-1 complexes of different composition has previously been described for AP-1 cooperation with the nuclear factor of activated T cells (NFAT) motif of the IL-2 enhancer (59–61).

RARα Impairs the Oct-2–dependent AP-1/OAP Function. Mapping of the Oct-2 domains required for TPA/calcium-induced activation function revealed that they overlapped the region required for negative regulation by RARα. This suggests that a focus for both activator and inhibitor signals may occur on a unique pathway. We show here that this pathway could be represented by the AP-1 complex. Abrogation of AP-1 binding to the OAP *cis*-element blunted the antagonistic activity of RAR. An indirect modulation of IL-2 promoter by RAR on AP-1/OAP function was suggested by the requirement for the receptor DNA–binding domain but not direct in vitro binding to the IL-2 octamer motif and by the ability of RARα to inhibit the AP-1 binding activity to the IL-2-OAP/OCT sequence in vitro. A similar mechanism has been proposed for RAR antagonism of collagenase AP-1 *cis*-element by an inhibition of the AP-1 complex DNA-binding activity in HeLa cells independently from direct RAR binding to DNA (18, 19, 62). We used a similar model to demonstrate inhibition of TPA-induced activation of collagenase AP-1 *cis*-regulatory activity by RARα in T cells. Therefore, although the in vivo binding of RAR to the OAP/OCT motif cannot be ruled out, the ability of the receptor to inhibit the OAP binding activity without direct DNA binding in vitro, presented here, is sufficient to suggest that RAR inhibits the functional synergism between AP-1 and Oct factors by preventing the binding of *jun* and *fos* to the OAP binding site. An appropriate genetic context for AP-1 regulation seems to be required, as RA does not interfere with the activity of the AP-1 complex found in the NFAT element of IL-2 enhancer (23, 63). Our data leave open, however, the question of the potential contribution of Oct factors in the negative regulation of AP-1 sites by RAR and their potential interaction with the receptor. In this respect, it should be noted that protein–protein interactions have been implicated in both the antagonism of Oct-2 function by glucocorticoid receptor (GR; 64) and the synergism between GR and Oct-1 (65). It is of further interest that the repression of the IL-2-OAP/OCT motif by RA, described here, is not without similarity to a recent report describing RAR-mediated interference with the activation of the calcitonin/calcitonin gene-related peptide enhancer by cell-specific helix-loop-helix and octamer proteins in combination (66). Another nuclear receptor (GR) has been described to cause a similar impairment of the functional cooperation between distinct *cis*-elements (AP-1 and NFAT) in the IL-2 enhancer (21).

Multiple Pathways of AP-1 Regulation: Implications for T Cell Development. Octamer-binding proteins containing homeodomains are developmentally regulated and involved in the cascade of events controlling morphogenic processes (14). As RA regulates octamer-binding proteins (9–12) and IL-2 is active in T cell development (25, 26), it is possible that RAR and octamer-binding factors might combine to modulate thymocyte differentiation through regulation of cytokine gene transcription. This hypothesis is consistent with our observation that RA can modulate T cell precursor development in an in vitro thymocyte differentiation system (67, 68). To extend this hypothesis, the data described here and elsewhere (30, 59–61, 63) suggest a central role for AP-1 sites in the functional organization of the IL-2 enhancer, which is composed of several regulatory modules, including an AP-1 site that potentially cooperates with another closely linked transacting factor–binding motif. This site may well be responsible for differential regulation by external stimuli. The role of AP-1 in regulating Oct-2 and mediating RAR activity would strongly suggest that it may also contribute to the T cell differentiation process. Support for this comes from observations that changes in the AP-1 pathway accompany the induction of T cell tolerance (69) and thymocyte ontogeny (70). As AP-1 responds to a broad range of activating stimuli, often unrelated to differentiation pathways, this transcription factor may provide signals that specify developmental programs on the basis of selective functional interactions with differentiation-related transacting factors.

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