Glucocorticoid Receptor-mediated Suppression of the Interleukin 2 Gene Expression through Impairment of the Cooperativity between Nuclear Factor of Activated T Cells and AP-1 Enhancer Elements

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Summary

The immunosuppressant hormone dexamethasone (Dex) interferes with T cell–specific signals activating the enhancer sequences directing interleukin 2 (IL-2) transcription. We report that the Dex-dependent downregulation of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and calcium ionophore–induced activity of the IL-2 enhancer are mediated by glucocorticoid receptor (GR) via a process that requires intact NH_2- and COOH-terminal and DNA-binding domains. Functional analysis of chloramphenicol acetyltransferase (CAT) vectors containing internal deletions of the −317 to +47 bp IL-2 enhancer showed that the GR-responsive elements mapped to regions containing nuclear factor of activated T cells protein (NFAT) (−279 to −263 bp) and AP-1 (−160 to −150 bp) motifs. The AP-1 motif binds TPA and calcium ionophore–induced nuclear factor(s) containing fos protein. TPA and calcium ionophore–induced transcriptional activation of homo-oligomers of the NFAT element were not inhibited by Dex, while AP-1 motif concatemers were not stimulated by TPA and calcium ionophore. When combined, NFAT and AP-1 motifs significantly synergized in directing CAT transcription. Such a synergism was impaired by specific mutations affecting the *trans*-acting factor binding to either NFAT or AP-1 motifs. In spite of the lack of hormone regulation of isolated *cis* elements, TPA/calcium ionophore–mediated activation of CAT vectors containing a combination of the NFAT and the AP-1 motifs became suppressible by Dex. Our results show that the IL-2-AP-1 motif confers GR sensitivity to a flanking region containing a NFAT element and suggest that synergistic cooperativity between the NFAT and AP-1 sites allows GR to mediate the Dex inhibition of IL-2 gene transcription. Therefore, a Dex-modulated second level of IL-2 enhancer regulation, based on a combinatorial modular interplay, appears to be present.

Immunosuppression by physiological and pharmacological agents (i.e., glucocorticoids, cyclosporin A, and FK-506) plays a key role in controlling immune reactions against endogenous or exogenous antigens and has been reported to be very effective in induction of transplantation tolerance (1). Most of the immunosuppressants affect the cascade of molecular events arising from the interaction of the antigen with the TCR or from other costimulatory agents that turn on the transcription of specific lymphokine genes such as IL-2 (1–6). IL-2 is the major growth factor for T lymphocytes, which is involved in T cell differentiation, functional activation, and proliferation (7, 8). Besides being widely used as therapeutical agents, glucocorticoid hormones are physiological immunosuppressants suggested to be involved in the control of immune and inflammatory hyper-reactivity during the stress response (9). We have previously reported that glucocorticoid hormones inhibit phorbol ester and calcium ionophore–induced transcription of the human IL-2 gene (10). We have also shown that such a glucocorticoid inhibition was not observed in fibroblasts expressing a transfected IL-2 gene, including 2.0 kb of 5′ flanking regulatory sequences, suggesting that the hormone interferes with T cell–specific activating

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signals (11, 12). T cell–specific signals switching on the IL-2 gene arise from interaction of the antigen with the TCR in combination with other costimulatory elements and subsequent activation of protein kinase C and intracellular calcium increase, which in turn activate the synthesis or the function of a set of *trans*-acting factors binding to consensus sequences in the 5′ flanking region of the gene (6, 13–21). Several putative enhancer *cis* elements have been identified in the −300 bp region of the gene, including two AP-1-like and octamer motifs, AP-3 and NFkB elements, a purine-rich region binding a less identified protein called nuclear factor of activated T cells (NFAT),[1] and a more recently reported CD28-responsive element (13–21). All of these *cis* elements are suggested to cooperate with each other to compose the overall IL-2 enhancer activity (14). These observations raise the question of how interference with such a transcriptional regulatory activity by immunosuppressive agents might occur. In this regard, glucocorticoid hormones are particularly interesting as agents potentially interfering with IL-2 enhancer *trans*-acting factors. Indeed, glucocorticoid hormones are known to control gene expression by activating intracellular receptors belonging to the steroid/thyroid hormone/retinoic acid receptor superfamily of nuclear *trans*-acting factors, which binds to specific consensus sequences provided of intrinsic enhancer properties (reviewed in reference 22). This suggests that the hormone-receptor complex might act at or proximal to the transcriptional steps involved in IL-2 gene expression. However, in contrast to results described for IL-2 gene inhibition by another immunosuppressant agent, cyclosporin A (23–25), the synthetic glucocorticoid hormone dexamethasone (Dex) has been recently reported not to affect the levels of the known IL-2 gene *trans*-acting factors (26). To study the molecular events implicated in the Dex-induced downregulation of the IL-2 enhancer, we delineated both the glucocorticoid receptor (GR) domains and the IL-2 *cis*-regulatory sequences mediating the hormone action. We report here that the GR, by a process that requires the presence of intact NH_2-terminal, COOH-terminal, and DNA-binding domains, selectively impairs the synergistic cooperativity of two distinct *cis* elements, the NFAT and AP-1 motifs, while it does not affect the enhancer activities of these isolated regulatory sequences. Our data suggest a novel mechanism of interference with a cooperative regulatory pathway of IL-2 enhancer *cis* elements involving a second level of transcriptional regulation based on combinatorial modular interplay.

Materials and Methods

Plasmids. The plasmid pIL2CAT contains the −575 to +47 bp IL-2 flanking region driving the expression of the chloramphenicol-acetyl-transferase (CAT) gene (27, 28). Plasmids (−317/+47) IL-2CAT and the internal deletion mutants of the IL-2 enhancer CAT vectors represented in Fig. 2 (a generous gift of Dr. G. R. Crabtree, Stanford University, Stanford, CA) have been described (14). CAT expression vectors containing concatemers of the IL-2 *cis* elements were constructed by inserting into the BamHI site of pBLCAT2 vector (29) (including the −105 bp fragment of the thymidine kinase [tk] promoter, missing the octamer motif and driving CAT gene expression) three copies of either the IL-2-NFAT site (fragment −294 to −261 bp of the IL-2 enhancer, 3x[NFAT]-tk-CAT), the IL-2-NFkB site (−210 to −192 bp, 3x[NFkB]-tk-CAT), the distal and proximal IL2-AP-1-like motifs (−188 to −170 bp and −160 to −139 bp, 3x[dAP-1]-tk-CAT and 3x[pAP-1]-tk-CAT, respectively), four copies of the proximal IL-2-octamer motif (fragment −96 to −66 bp, 4x[Oct]-tk-CAT), and two copies of a synthetic oligonucleotide spanning the −294/−265 bp NFAT region fused to the −160/−139 bp AP-1 motif (pNFAT-AP-1-tk-CAT). CAT expression vectors, containing concatemers of the mutated IL-2 *cis* elements (NFATm, AP1ml, and AP1m2) were constructed in a similar way after synthesis of corresponding oligonucleotides carrying the mutations indicated in figure legends. All of those multimers were constructed as synthetic oligonucleotides, including BamHI and BglII restriction sites at the 5′ and 3′ end, respectively. The plasmid p[−575/+47]tk-CAT contains one copy of the IL-2 enhancer −575 to +47 bp fragment cloned into the HindIII site of pBLCAT2 in the antisense orientation with respect to CAT gene transcription. GALV-AP-1-CAT contains six copies of the AP-1 motif-containing core enhancer element of the SEATO strain of the gibbon ape leukemia virus (GALV) LTR (30, 31). The plasmid MMTV-CAT (containing the LTR of the mouse mammary tumor virus) has been previously described (32). Wild-type (pRShGRα and HG0) and mutant (HG3, HG8, I37, I204, I422, and I582) GR expression vectors (33, 34) were provided by Drs. R. Evans (The Salk Institute, La Jolla, CA) and P. Chambon (INSERM U.184, LGME-CNRS, Strasbourg, France). The plasmid pCH110 (Pharmacia, Uppsala, Sweden) contains a functional lacZ gene, coding for β-galactosidase, under the transcriptional control of the SV40 early promoter.

Cell Culture and DNA Transfections. The human Jurkat T cell line was cultured in RPMI 1640 supplemented with 10% FCS and antibiotics (Flow Laboratories, Ayrshire, Scotland). Cells were treated with 30 ng/ml of 12-O-tetradecanoyl-phorbol-13-acetate (TPA), 1 μg/ml of A23187 (Sigma Chemical Co., St. Louis, MO), or 1.5 μg/ml of ionomycin (Calbiochem-Behring Corp., San Diego, CA) in the presence or in the absence of 1 μM dexamethasone (Sigma Chemical Co.).

Transfections of lymphoid cells were carried out by the DEAE dextran method (35) as described (10). Cells were cotransfected with various plasmids together with pCH110 (as internal control for transfection efficiency). 24 h after transfection, cells were treated with the drugs indicated above, and after further 24 h, cells were harvested and protein extracts prepared for the CAT and β-galactosidase assays.

CAT Assay. CAT assay was carried out as previously described (36) by incubating 50–100 μg of cell lysate protein with 0.1 μCi [^{14}C]chloramphenicol (sp act, 60 mCi/mmol; Amersham Corp., Arlington Heights, IL) in the presence of 9 mM acetyl-coenzyme A (Sigma Chemical Co.) for different times at 37°C. Acetylated and unacetylated chloramphenicol were separated by TLC and acetylation quantified by autoradiography and liquid scintillation counting.

β-Galactosidase Assay. A 20–30-μg sample of the same cell lysate prepared for analysis of CAT activity was diluted in 100 mM $NaPO_4$, 10 mM KCl, 1 mM Mg_2SO_4, 50 mM β-mercaptoethanol, pH 7.0; and β-galactosidase activity was determined spectro-

[1] *Abbreviations used in this paper:* CAT, chloramphenicol-acetyl-transferase; Dex, dexamethasone; GALV, gibbon ape leukemia virus; GR, glucocorticoid receptor; GRE, GR-responsive element; MMTV, mouse mammary tumor virus; NFAT, nuclear factor of activated T cells; tk, thymidine kinase; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

photometrically at 420 nm by the hydrolysis of *o*-nitrophenol-β-D-galactoside.

Gel Mobility Shift Assays. Cells were lysed in 10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.25 mM dithiothreitol, 0.5 mM PMSF, and nuclei were spun at 800 *g* and 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 dithiothreitol, 0.5 mM PMSF. Nuclear extract was cleared by centrifugation. Nuclear extracts (5 μg of protein) were incubated for 20 min at 25°C in 15 μl of reaction buffer containing 15 mM Hepes (pH 7.8), 70 mM NaCl, 0.5 mM dithiothreitol, 2% glycerol, 2.5 μg of poly(dI-dC), and 1 ng of ^{32}P-labeled IL-2-proximal AP-1 probe (−160 to −139) in the absence or in the presence of a 10- or 100-fold excess of cold wild-type AP-1 or mutated AP-1-ml or AP-1-m2 oligonucleotide competitors. The sequences of the oligonucleotides are as follows: AP1, AAATTCCAAAGAGTCATCAGAA; AP1m1, AAATTCCAAAGAactgTCAGAA; AP1m2, gggcTCCAAAGAGTCATCAGAA. Protein-DNA complexes were separated on 4% polyacrylamide gels with 22.5 mM Tris-borate (pH 8.0) and 1 mM EDTA buffer. In gel retardation experiments using fos- and β-galactosidase antibodies, nuclear extracts were preincubated with the antibodies for 15 min at 25°C before performing the DNA binding reaction described above. Affinity-purified antibody to fos peptide (amino acids 129–153) (37) was kindly donated by Dr. Michael Iadarola (National Cancer Institute, Bethesda, MD). Affinity-purified antibody against β-galactosidase was a gift of Dr. David Levens (National Cancer Institute) (38).

Results

Delineation of GR Domains Required for IL-2 Enhancer Repression. We have previously reported that Dex inhibits the TPA/calcium ionophore–induced transcriptional activation from the −575 to +47 bp region of the IL-2 gene driving the expression of the CAT reporter (10). Inhibition of TPA/calcium ionophore–induced activation of the IL-2 enhancer–CAT construct by Dex was strictly dependent on the presence of the cotransfected GR expression vector, suggesting that GR is involved in mediating the drug action on the IL-2 enhancer (10).

To delineate the GR domains involved in the repression of the IL-2 promoter activity, we studied the effects of several GR mutant expression vectors on the activity of the cotransfected pIL2-CAT. Fig. 1 shows that GR mutants HG8, I204, and to a lesser extent I37, carrying deletion (HG8) or mutations (I204 and I37) of the NH_2-terminal domain, respectively, have decreased Dex-mediated repression of the IL-2-CAT expression. Truncation of the COOH-terminal ligand binding domain (HG3) also impaired hormone-independent and -dependent repression activity (Fig. 1). Mutation of the receptor DNA-binding domain (I422) resulting in the disruption of the first zinc finger structure also reduced the hormone-dependent inhibition of pIL-2-CAT activity (Fig. 1). The effects of GR mutations on the inhibition of the IL-2 enhancer parallel those on the GR-responsive element (GRE) of the mouse mammary tumor virus (MMTV)-CAT vector, as far as the NH_2-terminal and DNA-binding domains are concerned (Fig. 1). In contrast, deletion of the COOH-terminal domain results in a hormone-independent constitutively active receptor *trans*-acting the MMTV-CAT expression (Fig. 1). Transcriptional activity of GRE by GR has been shown to require the DNA-binding and NH_2-terminal domains interacting with consensus sequence motif and other cooperating factors of the transcriptional machinery, respectively, being the COOH-terminal domain per se dispensable for *trans*-acting activity (33, 34, 39, 40). In contrast, our data show that the GR activity on the IL-2 enhancer, besides the NH_2 terminus and the DNA-binding region, also requires the COOH-terminal domain, suggesting that the receptor needs differential and/or potentially more complex interactions with the IL-2 transcriptional unit involving additional regions of GR.

Delineation of GR-responsive Elements in the IL-2 Enhancer. To delineate the *cis* regulatory elements of the IL-2 gene whose transactivation by TPA and calcium ionophore is inhibited by Dex, we studied the drug effect on the expression of transiently transfected CAT vectors in which reporter gene transcription was directed by 5′ deletion of the −575 to +47 bp region of the IL-2 gene or by the −317 to +47 bp fragment carrying several internal deletions. In agreement with

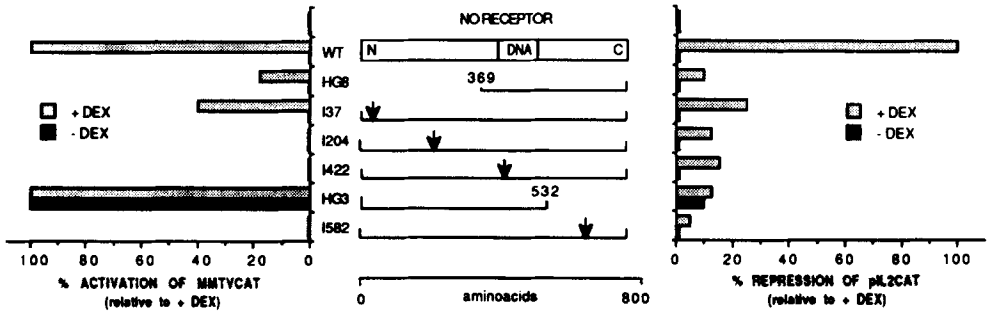


Figure 1. Repression of IL-2 enhancer activity by wild-type and mutant GR. Jurkat cells were transfected with 2 μg of either MMTV-CAT or pIL-2-CAT reporter plasmids in the absence or in the presence of espression vectors (1 μg) producing either wild-type (WT; *bar*) or various mutant GR (HG8, I37, I204, I422, HG3, I582; *solid lines*). In WT GR, N and C indicate NH_2 terminus and COOH terminus, and DNA indicates the DNA-binding domain. The I series of GR mutants is characterized by the insertion of three to four extra aminoacids in the positions indicated by the mutant name and shown by the arrows. The GR mutants I582 and HG3 are not able to bind the hormone. Half of the transfected cells were incubated in growth medium (*shaded bars*) and the other half in the presence of 1 μM DEX (*solid bars*). CAT activity was determined 24 h later and expressed as a percentage of either the activation of MMTV-CAT (*left*) or the repression of the TPA/calcium ionophore–induced IL-2-CAT expression (*right*) relative to wild-type GR-transfected cells. The results are representative of three experiments.

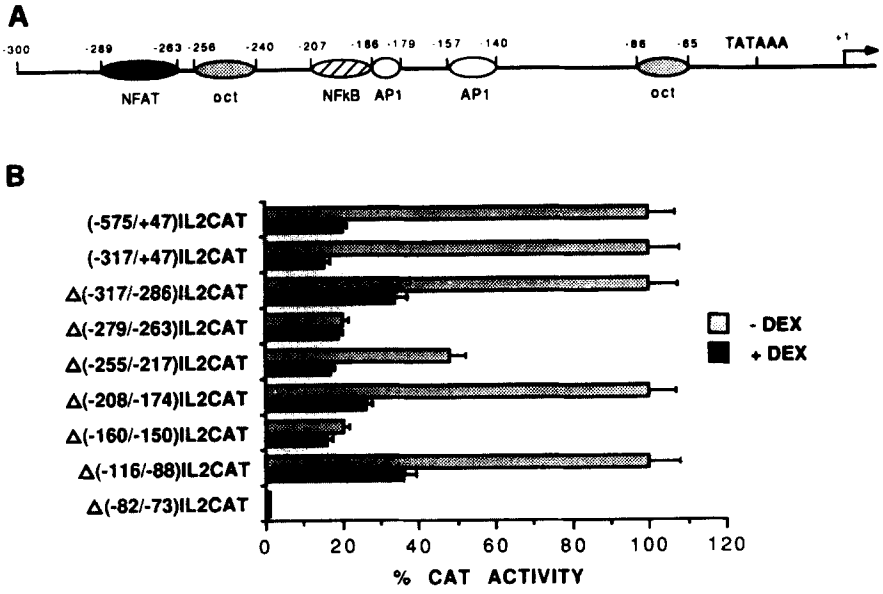


Figure 2. (*A*) Schematic representation of some of the positive transcriptional *cis*-regulatory elements in the 5′ flanking region of the IL-2 gene. *Cis* elements implicated in IL-2 gene transcription and the names of the putative nuclear binding proteins are indicated. Numbers indicate the base pair positions relative to the transcription start site. (*B*) Delineation of Dex-responsive elements by analysis of wild-type IL-2 enhancer ([−575 to +47 bp]IL-2-CAT or [−317 to +47 bp]IL-2-CAT), or mutants carrying the internal deletions indicated (Δ [−317/−286]IL-2-CAT, Δ [−279/−263]IL-2-CAT, Δ[−255/−217]IL-2-CAT, Δ[−208/−174]IL-2-CAT, Δ[−160/−150]IL-2-CAT, Δ[−116/−88]IL-2-CAT, Δ[−82/−73]IL-2-CAT) activating transcription of the CAT gene. 4 μg of the indicated plasmids plus 1 μg of wild-type GR expression vector and 1 μg of pCH110 were cotransfected into Jurkat cells, and 24 h later cells were treated with TPA and calcium ionophore as indicated in Materials and Methods in the absence or in the presence of 1 μM of DEX. The shaded and solid bars give the TPA/calcium ionophore–induced CAT activities (assayed 24 h later) in untreated (−DEX) and Dex-treated (+DEX) cells, respectively, expressed as the average (± SE) percent activity relative to the TPA/calcium ionophore–activatable [−575 to +47 bp]IL-2-CAT expression (2,550 ± 150 pmol/h/mg of protein and 30 ± 5 pmol/h/mg of protein in the presence and in the absence, respectively, of TPA/A23187).

previous reports (14), Fig. 2 shows that the main regulatory sequences of the IL-2 gene are located in the −317 to +47 bp region. The strongest enhancer *cis* elements of the IL-2 promoter map to the the NFAT, the proximal AP-1-like, and the proximal and the distal octamer motifs, since significantly decreased transcriptional activity was observed in constructs carrying internal deletion of those sequences. As previously reported (14), these deletion mutants still display transcriptional activity due to the function of isolated or cooperating residual *cis* elements. The Dex-responsive region was restricted to the −317 to +47 fragment of the IL-2 enhancer, since the (−317/+47) IL-2-CAT expression vector was inhibited to a similar extent as the (−575/+47) IL-2-CAT construct by Dex treatment (Fig. 2). Impairment of the Dex-induced inhibition of the transcriptional activity, as compared with the wild-type −317/+47 bp enhancer, was only observed in the mutants carrying limited (15 and 10 bp, respectively) and specific sequence deletions disrupting the NFAT site (Δ[−279/−263]IL-2-CAT) and the proximal AP-1-like motif (Δ[−160/−150)IL-2-CAT) (Fig. 2), and interrupting the binding of the cognate factors (14). This suggests that *cis* elements containing both NFAT and proximal AP-1-like motifs are responsive to the inhibitory action of Dex.

The IL-2-proximal AP-1-like Motif Binds an AP-1 Complex Containing fos Protein in Jurkat Cells. The deletion experiments described in Fig. 2 show that an AP-1-like motif appears to be involved in GR-mediated negative regulation. Since GR has been shown to inhibit the transcriptional activity of AP-1 *cis* elements by interfering with fos protein, a component of the AP-1 factor–DNA binding activity (41), we wanted to determine whether fos was present in the com-

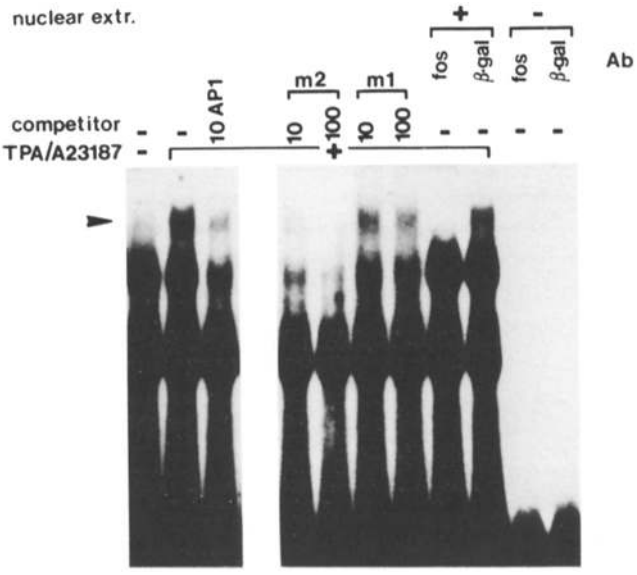


Figure 3. Gel retardation assay of nuclear extracts from untreated Jurkat cells or cells treated for 2 h with TPA (30 ng/ml) and A23187 (1 μg/ml). Nuclear extracts (5 μg) were incubated as described in Materials and Methods with a labeled (−160 to −139) IL-2-AP-1 probe in the absence or in the presence of a 10-fold or a 100-fold excess of either unlabeled wild-type IL-2-AP-1 or mutated AP-1-m1 (m1) or AP-1-m2 (m2) oligonucleotides. AP-1-m1 and AP-1-m2 carry mutations inside and outside the AP-1 target sequence, respectively. The figure also shows the nuclear factor–DNA complex formed in the presence of antibody (Ab) against fos or β-galactosidase (β-gal) antigens. Arrowhead indicates the inducible AP-1 protein–DNA complex.

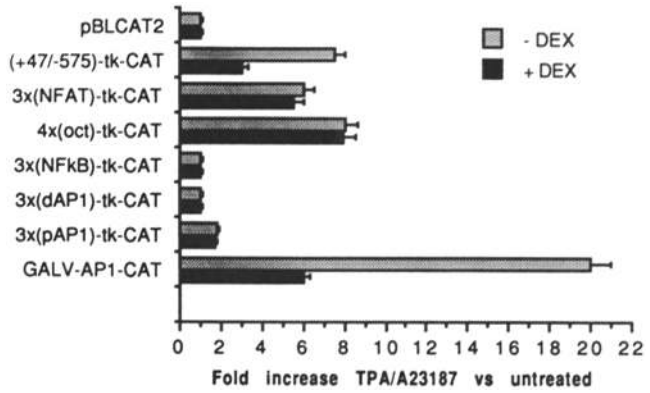


Figure 4. Effect of Dex treatment on the ability of multimers of IL-2 *cis* elements to activate the tk promoter-CAT transcriptional unit. 4 μg of the indicated IL-2-derived tk-CAT expression vectors and pGALV-AP-1-CAT were transfected into Jurkat cells. After 24 h, cells were treated with TPA/calcium ionophore and Dex as indicated in Fig. 2. Results are expressed as the average (± SE) fold increase of the CAT activity observed in TPA/calcium ionophore–treated relative to untreated cells in the absence (−DEX) or in the presence (+DEX) of 1 μM Dex treatment.

plex formed by the IL-2-proximal AP-1-like motif and nuclear extract from phorbol ester/calcium ionophore–activated Jurkat cells. Fig. 3 shows that an antibody against fos protein was able to inhibit the binding of nuclear factor(s) to the IL-2 AP-1-like motif, while an unrelated antibody was uneffective, suggesting the presence of fos or a fos-related antigen in that complex. Oligonucleotides carrying mutations within the AP-1 target sequence (m1) do not compete for the binding of nuclear factor to the wild-type motif, while a mutation outside the AP-1 sequence (m2) resulted in an actively competing oligonucleotide (Fig. 3). These data provide evidence that a typical AP-1 factor binds to the IL-2-proximal AP-1 motif in Jurkat cells.

Dex Impairs the Synergism between the NFAT and AP-1 Motifs. To further delineate the *cis* elements responsive to Dex, we studied the ability of the drug to affect the TPA/calcium ionophore–activated transcription of the tk promoter-driving CAT gene directed by multimers of the NFAT, proximal octamer, distal and proximal AP-1, and NFkB binding sites. Fig. 4 shows that the transcriptional activity of NFAT and octamer motifs were significantly enhanced by TPA and calcium ionophore treatment. In contrast, neither NFkB nor AP-1 elements were significantly enhanced by TPA and A23187. The incapability of the NFkB-CAT construct to respond to TPA/A23187-mediated activation together with the failure of the internal deletion of the NFkB site to decrease the transcriptional activity of the whole enhancer (reference 14 and Fig. 2) suggest that this element is not functional. This is however in contrast with the reported decreased enhancer activity caused by point mutations of the NFkB site affecting its factor binding capability (15) and by the ability of NFkB concatemers to enhance transcription from heterologous promoter in response to phorbol ester and PHA in murine EL4 cells (17). All of these data, taken together, suggest that determined steric configuration of the IL-2 enhancer and/or cell-specific microenvironment is required for the proper functioning of the NFkB element. In contrast to the IL-2-AP-1-CAT construct, another AP-1 motif derived from the GALV LTR (GALV-AP-1-CAT) was significantly enhanced by TPA and calcium ionophore (Fig. 4). Dex was not able to inhibit the expression of any of the IL-2-derived multimeric constructs either in the absence or in the presence of TPA and calcium ionophore, except for the −575 to +47 bp region of the IL-2 enhancer driving the CAT gene through the tk promoter (p[+47/−575]tk-CAT) (Fig. 4). Interestingly, the GALV-AP-1-CAT expression was significantly inhibited by Dex, as previously reported (10). In spite of the involvement of the NFAT site in mediating Dex action on

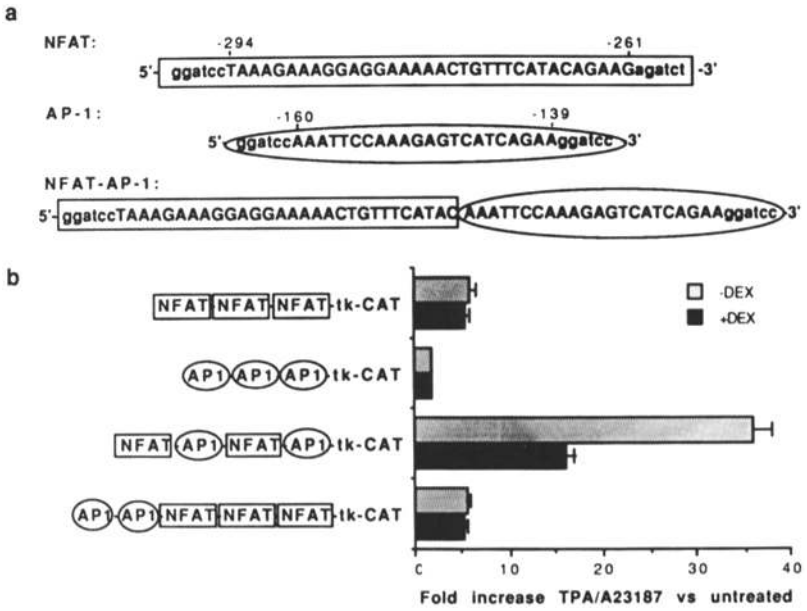
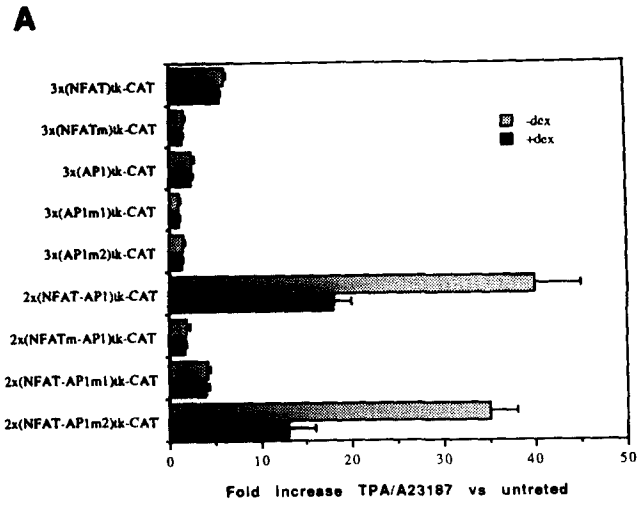


Figure 5. DEX inhibits the synergism between the NFAT and the AP-1 motifs in directing transcription of CAT gene from the tk promoter. Different combinations of synthetic oligonucleotides spanning the NFAT and proximal AP-1 motifs (indicated in *a*) were cloned into the BamHI site in front of the fusion gene tk-CAT. (*a*) The sequences of the NFAT and AP-1 oligonucleotides corresponding to the concatemers shown in the first, second, and fourth rows of *b* and of the combined NFAT-AP-1 oligonucleotide indicated in the third row. Lowercase letters indicate the BamHI and BglII linker restriction sites. 4 μg of the indicated tk-CAT constructs plus 1 μg of wild-type GR expression vector and 1 μg of pCH110 were transfected into Jurkat cells, and 24 h later cells were treated as indicated in Fig. 2. (*b*) Results are expressed as the average (± SE) fold increase of the CAT activity observed in TPA/calcium ionophore–treated relative to untreated cells in the absence (−DEX) or in the presence (+DEX) of 1 μM Dex treatment.

the intact IL-2 enhancer suggested by experiments shown in Fig. 2 using internal deletion mutants, hormone was unable to inhibit the transcriptional activity of homo-oligomers of this *cis* element (Fig. 4). This suggests that Dex does not affect the transcriptional activity of the NFAT motif per se, implying that the hormone inhibitory action requires the cooperation of other *cis* elements or sequences adjacent to the NFAT site that are lost or disrupted in the NFAT-tk-CAT construct. Since deletion of the proximal AP-1 motif also impaired Dex sensitivity of the IL-2 enhancer (Fig. 2), we speculated that cooperativity between the NFAT and AP-1 sites was required for conferring Dex-induced downregulation of the IL-2 promoter. For this purpose, we first tested the hypothesis that transcriptional cooperativity would exist between the two *cis* elements by constructing CAT vectors containing different combinations of NFAT and proximal AP-1 elements. Fig. 5 shows that introduction of two AP-1-like elements 5′ to the NFAT site does not further enhance the capability of the NFAT motif to respond to TPA and calcium ionophore. In contrast, although the proximal AP-1 site is unable to be significantly activated by TPA and A23187 on its own, a dimer of an oligonucleotide spanning a NFAT site and a proximal AP-1 motif in the 5′ to 3′ configuration shows a significant increase in the capability to respond to TPA/A23187 with respect to the NFAT-alone tk-CAT construct (Fig. 5). To study whether the transcriptional synergism between the two elements involved sequences binding NFAT and AP-1 factors, respectively, we introduced four base pair substitutions in the NFAT and AP1 motifs (NFATm and AP1m1, respectively), which specifically interrupted the factor binding ability (Fig. 3 and references 20 and 42). Mutation of the NFAT target sequence (NFATm) significantly reduced the ability of either enhancing the TPA/A23187-induced CAT transcription from NFAT homo-oligomers (3xNFATm-tk-CAT) or synergizing with wild-type AP-1 (3x[NFATm-AP-1]tk-CAT) (Fig. 6). Similarly, mutation of the AP-1 target sequence (AP-1m1) abolished the synergism with the wild-type NFAT element (2x[NFAT-AP-1m1]tk-CAT) (Fig. 6), while mutation outside the AP-1 target sequence (AP-1m2), which did not affect the binding of nuclear factor from TPA/A23187-induced cells (Fig. 3 and reference 20), was unable to do so (2x[NFAT-AP-1m2]tk-CAT, Fig. 6). All of these data taken together suggest that the proximal AP-1 motif synergizes with the NFAT site as far as its transcriptional activation by TPA and A23187 is concerned. Furthermore, this also suggests that this cooperativity requires a specific steric configuration of the AP-1 element relative to the NFAT motif, as physiologically occurring in the intact IL-2 enhancer. Interestingly, the proximal AP-1 element is able to confer Dex-induced downregulation to the NFAT motif, since Dex was able to significantly inhibit the transcriptional activity of $(NFAT\text{-}AP\text{-}1)_2$-tk-CAT construct, while not affecting the expression of NFAT-tk-CAT (Fig. 5). Mutations specifically affecting the NFAT (NFATm) and AP-1 (AP-1m1) binding sites abolished the Dex-induced downregulation of combined concatemers, while a mutation of the AP-1 motif outside the AP-1 target sequence (AP-1m2) retained the hormone sensitivity when this mutated AP-1 element was combined with the wild-type NFAT motif (Fig. 6).



B

NFAT: 5′-TAAAGAAAGGAGGAAAAACTGTTTCATACAGAAG-3′

NFATm: 5′-TAAAGAAAGGAGaAAAAAaTtTTTaATACAGAAG-3′

AP1: 5′-AAATTCCAAAGAGTCATCAGAA-3′

AP1m1: 5′-AAATTCCAAAGAactgTCAGAA-3′

AP1m2: 5′-gggcTCCAAAGAGTCATCAGAA-3′

Figure 6. Mutations of NFAT and AP1 binding sites abolish transcriptional cooperativity and Dex-regulation. Different combinations of synthetic oligonucleotides spanning the wild-type (NFAT and proximal AP-1) or mutated (NFATm, AP-1-m1 and AP-1-m2) motifs were constructed as described in Fig. 5. (*B*) The sequence of the oligonucleotide used. Lowercase letters indicate the mutated nucleotides. The AP-1 motif is underlined. 4 μg of the indicated tk-CAT constructs plus 1 μg of wild-type GR expression vector and 1 μg of pCH110 were transfected into Jurkat cells, and 24 h later cells were treated as indicated in Fig. 2. (*A*) Results are expressed as the average (± SE) fold increase of the CAT activity observed in TPA/calcium ionophore–treated relative to untreated cells in the absence (+DEX) or in the presence (+DEX) of 1 μM Dex treatment.

Discussion

Several enhancers are known to be composed of multiple sequence motifs functioning cooperatively or noncooperatively and binding cellular *trans*-acting factors involved in eliciting the transcriptional machinery (43, 44). Genetic analysis of the transcriptional enhancer of the Siminan Virus 40 allowed the identification of distinct classes of enhancer motifs (enhansons) characterizing multiple levels of functional organization (43, 45–47). Combination of isolated enhansons makes up enhancer function as a result of the synergistic action of the single *cis* elements provided of strong (type I, III, and IV protoenhancers) or very weak (type II protoenhancer) enhancer activity on their own (45, 46). The IL-2 enhancer is also composed of isolated *cis* elements binding cognate *trans*-acting factors and cooperating in making up the overall enhancer activity in response to the multiple signals arising from the extracellular microenvironment (14–20). In-

deed, deletion of each one of these *cis* elements dramatically decreases the overall enhancer activity of the −317 to +47 bp IL-2 region leaving however a transcriptional activity due to the presence of residual *cis* elements (14, 15, 19). However, how the IL-2 enhansons cooperate each other and whether such a cooperativity represents a level at which transcriptional modulation by extracellular agents might occur is still poorly understood. These questions have been specifically addressed in this paper. Here we show that the IL-2 enhancer is composed of at least a type II proto-enhancer unit represented by the combination of a strong enhancer element on its own (the NFAT site) and a very weak one (the proximal AP-1 motif). This is supported by the observation that, in spite of being a highly synergistic element in the whole enhancer, as demonstrated by the decrease of the overall enhancer activity caused by its internal deletion (reference 14 and Fig. 2), the AP-1 motif is different in its intrinsic enhancer activity from the NFAT element. In fact, while tandem repeats of the NFAT site are provided of transcriptional *cis*-regulatory activity on their own, homo-oligomers of the AP-1 motif are not. Nevertheless, the AP-1 motif combined with the NFAT site synergistically generates enhancer activity higher than that of NFAT homo-oligomers. Interestingly, a specific stereoalignment or spacing between the two elements appears to be required for optimal cooperativity, since no synergism was observed when tandem repeats of the AP-1 motif were introduced 5′ to the NFAT site instead of 3′, as physiologically occurs in the IL-2 enhancer.

An interesting question that rose from the combinatorial functional organization of the IL-2 enhancer deals with the possibility that a hierarchical order of preferential cooperativities among selected enhansons occurs. By studying the interference of the activation of the multiple *cis* elements of the IL-2 enhancer by the GR, our results suggest that such a preferential cooperativity between NFAT and AP-1 elements does occur, since those two motifs compose a regulatory pathway selectively affected by the GR. Indeed, we have observed that both sequences containing the NFAT and AP-1 motifs, when located in the physiologic genetic context of the whole IL-2 enhancer, are responsive to GR-mediated negative signals and that the regulation of the isolated NFAT element by GR is only conferred by the involvement of the AP-1 motif. The molecular mechanism by which GR interferes with AP-1-NFAT cooperativity needs to be elucidated. Negative regulation of gene expression by GR might be due to altered synthesis of specific *trans*-acting factors (48–52). Such a mechanism does not appear to be responsible for the downregulation of the IL-2 enhancer by GR, since expression of *trans*-acting factors binding to both NFAT and AP-1 motifs have recently been reported to be unaffected by Dex (26). Synergistic action of steroid hormone receptors with homologue or heterologue *trans*-acting factors has been reported to give rise to enhancer positive cooperativity (53, 54). Such a positive cooperativity is suggested to be mediated by the binding of the receptor to its cognate enhancer motif and subsequent protein-protein interactions with *trans*-acting factors and/or other components of the transcriptional machinery. A cooperative mechanism might also account for negative gene regulation by steroid receptors, since suppression of transcription via either receptor-mediated competition with positive *trans*-acting factors for binding to overlapping *cis* elements or interference with such positive factor function through protein-protein interaction have been reported (51, 52, 55, 56). GR has been recently reported to bind to the jun/fos/AP-1 *trans*-acting complex and to inhibit its function upon the collagenase AP-1 motif (41, 51, 52, 56). Dex is also able to inhibit the TPA-inducible activity of another AP-1 motif derived from the GALV LTR (this paper and reference 10). A similar antagonism between GR and the AP-1 element of the IL-2 enhancer might also occur. Indeed, the AP-1 motif of the IL-2 enhancer has been shown to bind a purified AP-1 factor from HeLa cells (17). Furthermore, collagenase AP-1 motif and the AP-1 element of the IL-2 enhancer cross-compete for binding of the same set of phorbol ester and calcium ionophore–inducible nuclear factors in Jurkat cells (21). Direct evidence that (−160 to −150 bp) IL-2-AP-1 motif binds AP-1 complex in activated Jurkat cells is given in this work by the observation of the presence of fos or a fos-related antigen in the nuclear factor(s)-DNA complex. Mapping of the GR domains involved in the functional inhibition of the transcriptional activity of the IL-2 enhancer reveals that the NH_2-terminal, COOH-terminal and DNA-binding domains are required for optimal repression. Requirement of the same GR domains has also been described for the interaction with the jun/AP-1 complex in the regulation of the collagenase AP-1 motif (56). Although the composition of the *trans*-acting factors binding to the IL-2-AP-1 motif and the nature of the potential interaction of the GR with both or either these factors and/or the cognate *cis* elements remain to be further studied, the GR-mediated downregulation of the IL-2 *cis*-regulatory sequences we have reported here appears to be quite peculiar. In fact, so far, GR has been described to interfere with the positive factor that transacts elements provided of transcriptional activity on their own, resulting in a subtraction of an enhancer unit from the overall regulatory region (class A, C, or D enhansons; 51, 52, 55, 56). The GR-mediated downregulation of the AP-1 motif reported here suggests a novel mechanism of transversal negative regulation by steroid receptors in which receptor sensitivity is conferred to flanking *cis* elements provided of enhancer properties on their own through cooperativity with a class B enhanson (AP-1 motif), which is the presumable element involved in GR-mediated signals.

Switching off or on the activity of isolated *cis* elements so far has also been reported as the major mechanism involved in the modulation of the IL-2 enhancer. For instance, cyclosporin A and other immunosuppressants that inhibit peptidyl-propyl *cis-trans* isomerases have been shown to decrease the transcriptional activity of homo-oligomers of selected IL-2 *cis* elements (23–25). A similar mechanism recently has been reported for another member of the steroid hormone receptor superfamily, the retinoic acid receptor, which inhibits the enhancer activity of the IL-2-octamer motif (57). Here we have provided evidence of a second level of modulation of the IL-2 enhancer function in which hormone-mediated decreased transcriptional activity is strictly dependent on the

heterologous combination of distinct *cis* elements. This would dramatically amplify the spectrum of agents and corresponding transducing molecular pathways activating defined IL-2 enhansons, which would in this way be able to interfere with the overall IL-2 gene expression on the basis of complex combinatorial modular interplays.

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