Negative Regulation of Interleukin 2 Transcription by the Glucocorticoid Receptor

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Summary

Glucocorticoid-dependent transcriptional enhancement is known to occur through the interaction of the glucocorticoid receptor (GR) with specific DNA response elements. In contrast, negative regulation of gene expression by this class of hormone is less well understood. Glucocorticoids are potent immunosuppressive agents acting primarily by inhibiting T lymphocyte activation and lymphokine production. Interleukin 2 (IL-2) gene expression, a critical early event during T lymphocyte activation, is inhibited in glucocorticoid-sensitive cells by hormone treatment. We have studied the mechanism of this inhibition. In transgenic mice carrying c-*myc* linked to the IL-2 enhancer, mitogen-induced expression of the transgene is inhibited by concurrent glucocorticoid treatment, while a similar transgene construct driven by three copies of the binding site for nuclear factor of activated T cells is not inhibited. Cotransfection experiments into glucocorticoid-insensitive jurkat cells show that the NH_2 terminus of the glucocorticoid receptor is dispensible for inhibition of the IL-2 enhancer but that an intact DNA binding domain, although not necessarily binding to DNA, is required. Hybrid GRs containing the DNA binding domains of either the estrogen receptor (ER) or thyroid receptor, as well as the entire wild-type ER, all function as repressors of the IL-2 enhancer. We have localized the site of inhibition to two sequences located in the proximal half of the enhancer. These sequences bind a similar, if not identical, inducible nuclear factor that has biologic characteristics that distinguish it from AP-1. The mechanism of IL-2 inhibition likely involves direct interactions between the GR and this factor.

Glucocorticoid hormones have widespread effects in a variety of target tissues. Many of these effects, both positive and negative, are a result of specific alterations in gene expression. The positive actions are mediated through the interaction of hormone with specific soluble receptors present in target cells. The glucocorticoid receptor (GR)[1] is a member of a superfamily of ligand-activated hormone receptors that function as transcriptional modulators (1). Hormone receptor complexes bind to specific sequences (glucocorticoid-responsive elements [GREs]) in the 5′ flanking regions of target genes and alter transcriptional activity (2). A large number of genes are transcriptionally activated by the GR; and their GREs, effectively acting as inducible enhancers (3), have a strong sequence conservation. Although there is a considerable body of knowledge about how glucocorticoids activate gene expression, the major clinical uses exploit their growth inhibitory, immunosuppressive, and antiinflammatory actions, which are, by comparison, considerably less well understood. The antiinflammatory effects have been attributed to the induction of a set of proteins called lipocortins, which inhibit phospholipase A2 (4), or alternatively, to direct inhibition of expression of inflammatory mediators such as IL-1 (5) or secreted proteases such as collagenase (6) and stromelysin (7). In addition, glucocorticoids participate in negative feedback loops, thus inhibiting expression of proopiomelanocortin (POMC) (8), and have adverse effects on reproductive function by negative regulation of a variety of genes including prolactin (9), proliferin (10), and the chorionic gonadotropin α subunit (11). Negative transcriptional regulation of these genes has been postulated to occur via the interaction of the GR with negative GREs (nGREs) (9) located in their 5′ flanking regions. These nGREs appear to be distinct from and not as highly conserved as GREs, and have been proposed to overlap binding sites for transcriptional activators on the DNA. Recent studies, however, have described a mechanism whereby the GR interferes with the activity of the transcription factor AP-1 by direct protein-protein interactions (12–15).

Disease states where T cell– and cytokine-mediated tissue damage predominates, including many examples of chronic inflammation, delayed hypersensitivity, and transplant rejec-

[1] *Abbreviations used in this paper:* GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; LS, linker scanner; NF-AT, nuclear factor of activated T cells; nt, nucleotide; OAP, octamer-associated protein; Tag, T antigen.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/92/05/1235/11 $2.00
Volume 175 May 1992 1235–1245

tion, are also effectively treated with glucocorticoids. We have studied the intracellular signaling pathways operative during the early phases of T lymphocyte activation using the IL-2 gene as a model system. T lymphocytes are activated by contact with specific antigen in conjunction with MHC molecules on the surface of APCs (for review, see reference 16). In addition, an accessory signal provided by APCs in the form of IL-1, IL-6, or IL-7 is needed for T cell activation (17–19). Critical events that occur during primary immune responses are proliferation of antigen-specific T cells, differentiation, and cytokine production. IL-2 plays a central and early role in these processes, and inhibition of IL-2 production is therefore likely to mediate a large number of glucocorticoid effects on immune function. Previous studies have shown inhibition of IL-2 production (20), IL-2 mRNA accumulation (21), and IL-2 transcription (22) after glucocorticoid treatment of T cells. Since addition of IL-2 to cultures of T cells stimulated in the presence of glucocorticoids overcomes the inhibition of T cell activation, it is likely that the major effect of the drug is to block IL-2 production. To investigate the mechanism of transcriptional repression of IL-2, we have examined GR activity on functional regions of the IL-2 enhancer. In addition, we have studied functional domains of the GR needed for repression. Our results help define a mechanism for glucocorticoid inhibition of IL-2 expression.

Materials and Methods

Cells and Culture. Human jurkat cells and mouse LBRM T cell lymphoma cells were grown in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2/95% air humidified atmosphere.

Plasmids. Expression vectors for the mouse glucocorticoid receptor and derivatives have, for the most part, been described as follows: SV2Wrec and SV2NB5'rec (NB5') (23); Δ395 and Δ575 (24); pGE and pGE9 (ΔEG) (25). The hybrid receptor (TR) containing the DNA binding domain of the human TR between the first and last conserved cysteine residues inserted into the vector pSV20.3Xrec was a gift of Lindsay Hinck (Syntex Research, Palo Alto, CA). The construct pJ3MOR encoding the mouse estrogen receptor has been described (26). The plasmid pSV2neo has been described (27). Reporter constructs based on CAT: pIL-2CAT, linker scanner (LS)-generated internal deletion mutants of the IL-2 enhancer LS5 (ID 279/263), LS29 (ID 208/174), LS1 (ID 140/120), LS20 (ID 153/121), and the duplicated proximal IL-2 enhancer construct DSCAT (BB), have all been previously described (28). MMTVCAT has been described (23) as has RSVCAT (29). Nuclear factor of activated T cells (NF-AT) p22-6 was constructed by Verweij (30) by inserting three copies of the NF-AT binding site (IL-2 sequences −286 to −257) into the XhoI site of p22-6. p22-6 contains a minimal IL-2 promoter (sequences −294 to −72 deleted from pIL2CAT). Oct p22-6 was similarly constructed by insertion of four copies of the Oct1/OAP binding site (−65 to −94) into the XhoI site of p22-6. The plasmids APCAT and the *lacZ*-based reporter constructs NFIL-2BZH, NFATZH, KBZH, and pIL-2ZH have been described (31). OctZH was constructed by Jeff Riegel (Howard Hughes Medical Institute, Stanford, CA) by inserting four copies of the Oct1/OAP site (−65 to −94) in place of the three NF-AT binding sites in NFATZH.

Transfections and Stimulations. Jurkat cells growing at a density of not greater than 10^6/ml were harvested by low-speed centrifugation and resuspended in growth medium at a density of 3 × 10^7/ml. Plasmid DNAs, 10 μg of reporter construct, and 10 μg of receptor construct or pSV2neo in a total volume of 20 μl 10 mM Tris (7.5), 1 mM EDTA, were added directly to 300 μl (~10^7 cells) of the concentrated cell suspension. This mixture was electroporated in a 0.4-cm cuvette with a gene pulser (Bio-Rad Laboratories, Richmond, CA) using 250 V and 960 μF capacitance. Cuvettes were then gently agitated for 5 min and the cell mixtures resuspended in 12 ml complete growth medium.

Stimulations were initiated after 40 h of growth using 2 μM ionomycin (Calbiochem-Behring Corp., San Diego, CA) dissolved in DMSO and 10 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) dissolved in ethanol plus or minus 10^{-6} M dexamethasone or 10^{-7} M estradiol dissolved in ethanol. Stimulations were allowed to proceed for 8 h before analyzing reporter gene activity. To eliminate errors due to differences in transfection efficiency, different stimulation conditions for the same reporter/receptor combination were done on aliquots of cells taken from the same electroporation cuvette. The relevant data for these studies are the extent to which inducible reporter gene activity is inhibited by hormone treatment. The inclusion of a construct to normalize for transfection efficiency has no effect on these data and was therefore not included except in experiments where the constitutive receptor, Δ575, was compared with the wild-type GR. These transfections included 2 μg of RSVCAT as an internal control.

CAT/B-Galactosidase Assays. CAT assays were carried out essentially as described (32). Acetyl coenzyme A was from Pharmacia Fine Chemicals (Piscataway, NJ) and ^{14}C-chloramphenicol (57 mCi/mmol) from Amersham Corp. (Arlington Heights, IL). CAT assays were quantitated directly using a radionucleotide imaging system (Ambis Systems, San Diego, CA). β-galactosidase activity produced from the *lacZ* reporter gene was measured in triplicate 200-μl aliquots of transfected cells using 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co.) as a substrate as described (31), except that 150 μl of reaction mixture containing 0.6 mM substrate was added to the cell pellets, and incubations were allowed to proceed for 3 h at 37°C before stopping. Fluorescence at 460 nm was measured using 355-nm excitation with a Titertek fluoroscan II (Flow Labs, McLean, VA).

Ribonuclease Protections. Transgenic mice used in these studies were established by C. Verweij (Central Laboratory of the Netherlands Red Cross) and E. Lacy (Sloan-Kettering Memorial Institute). The T antigen carrying line, Tag8, has been described (30). This line expresses SV40 T antigen (Tag) under the control of three NF-AT binding sites linked to the minimal IL-2 promoter. Tag transcripts are detected in spleen, thymus, and bone marrow cells only after stimulation with ionomycin and PMA. A second transgenic line was established that carries a single copy of the human *c-myc* gene driven by the human IL-2 promoter/enhancer sequences −586 to +47. The line used (no. 17) expresses *c-myc* transcripts in spleen and thymus only after mitogen stimulation (C. Verweij, E. Lacy, and G. Crabtree; unpublished results).

Ribonuclease protection probes used were as follows. To detect endogenous murine IL-2 transcripts, a 160-bp SacI/HindIII fragment excised from the plasmid pCD-IL-2 (provided by Frank Lee, DNAX, Palo Alto, CA) was cloned into the SacI/HindIII sites of pBluescript KS(+) (Stratagene, La Jolla, CA). This construct was linearized with SacI and a ^{32}P-labeled transcript synthesized from the T3 promoter. The resulting 200-nucleotide (nt) probe protects a 160-nt fragment. To detect the *c-myc* transgene, a 350-nt probe was synthesized from the SP6 promoter using the plasmid pSP65Gal (33) linearized with PstI. This probe protects the 47 nt

of human IL-2 sequence present in the transgene message. To detect the Tag transgene, a 272-bp PstI/HindIII fragment of the SV40 Tag gene was cloned into pBluescript KS (−). This construct was linearized with HindIII and used as a template for the synthesis of a 330-nt riboprobe from the T7 promoter. This probe protects 272 nt of the Tag message. Spleen cells from 6–8-wk-old mice were prepared as described (30) and stimulated for 3 h with 2 μM ionomycin plus 10 ng/ml PMA. Dexamethasone (10^{-6} M) was added at the indicated times before or during the stimulation. Ribonuclease protection using 5 μg of RNA per sample was performed as described (31, 34). For the Tag mice, RNA samples were incubated with both the mouse IL-2 and the Tag probes simultaneously. RNA samples from the *c-myc*-expressing mice was incubated with both the IL-2 and SP65Gal probes so that both protected bands appear in the same lane. Quantitation was done using the Ambis system as described for CAT assays.

Electrophoretic Mobility Shift Assays. Small scale nuclear extracts from jurkat and LBRM cells stimulated for 3 h with 10 ng/ml PMA plus 2 μM ionomycin plus or minus 10^{-6} M dexamethasone were made as described (33). Gel mobility shift assays used the following oligonucleotides as probes, competitors, or both: AP-1, tcgaGTGACTCAGCGCGtcga containing the AP-1 binding sequence from the human metallothionein enhancer (35); NFIL-2B, tcgaCAATTCCAAAGAGTCATCAGAAGAGGACtcga containing IL-2 sequences −134 to −159; octamer-associated protein (OAP), gtCTTTGAAAATATGTGTAATATgt containing IL2 sequences −76 to −100; Oct/OAP, gatcTTTGAAAATATGTGTAATATGTAAAACATTTTGgatc containing IL-2 sequences −65 to −97; NF-AT, gatcAAGGAGGAAAAACTGTTTCATGgatc containing IL-2 sequences −268 to −288. nt in lower-case letters above represent 5′ overhanging ends that are filled in with Klenow when the double-stranded oligonucleotides are labeled for probes. Otherwise, the overhangs are not filled in.

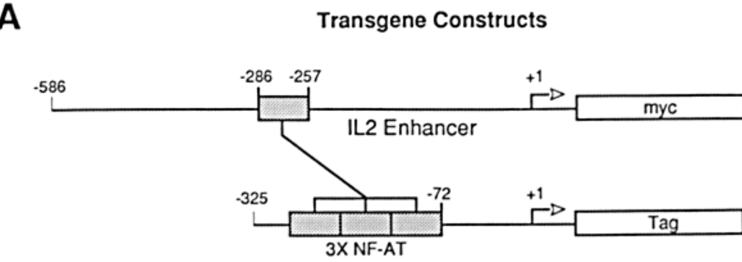
Gel mobility shifts were done as described (33) using 10 μg of jurkat or LBRM extract. In all cases, proteins were preincubated with buffer, nonspecific competitor, and specific competitor oligonucleotides where noted for 15–20 min before addition of the labeled probe. Competitor oligonucleotides were used at 150–200-fold molar excess over labeled probes. Incubations with labeled probes were done in a 20-μl volume containing 10 mM Tris (7.5), 50 mM NaCl, 0.5 mM EDTA, 5% glycerol, and 1.7 μg of poly dIdC. After 60 min at room temperature, samples were loaded on 4.5% polyacrylamide gels and run at room temperature.

Results

Dexamethasone Inhibits Both Endogenous IL-2 and an IL-2 Enhancer-driven Transgene. Previous studies have shown that negative regulation of IL-2 expression in the lymphoblastoid T cell line jurkat is, at least in part, transcriptional, and IL-2 sequences within 600 bp upstream from the start of transcription are implicated in mediating this repression (22). For our studies we felt it necessary to determine first whether this negative regulation could also be observed in splenic lymphocytes. We investigated this in transgenic mice carrying a *c-myc* transgene driven from human IL-2 sequences −586 to +47 (E. Lacy and G. Crabtree, unpublished results). In addition, we wanted to test, under similar circumstances, whether a construct responsive to only a single transcription factor essential for IL-2 gene activation, NF-AT, would be sensitive to negative regulation by the glucocorticoid dexamethasone. This was tested in a second transgenic line containing SV40 Tag driven by a minimal IL-2 promoter and a trimer of NF-AT binding sites (−257 to −286) (30) (Fig. 1 *A*). These mice express properly initiated messages in lymphoid tissues only after mitogenic stimuli, thus mimicking closely the expression of endogenous IL-2. The results shown in Fig. 1 *B*, lanes *1–7*, indicate that the expression of the IL-2/*c-myc* fusion gene follows the expression of the endogenous murine IL-2 exactly. This confirms that sequences within 600 bp of the start of transcription are sufficient for both activation-specific expression and dexamethasone-induced repression of IL-2 gene activity. Repression of IL-2 message is not complete at 3 h (40–54% inhibition for both the IL-2 and IL-2/*c-myc* messages when dexamethasone is given 1 h before or at the time of activation), while reporter gene activity in transient expression assays can be much more extensively inhibited (see below). This partial inhibition of IL-2 message production is evident even when looking at nuclear run-on transcription (22). Some repression can still be observed when dexamethasone is given well after the time when IL-2 message is first detectable and after the time (∼45 min) when inhibitors of protein synthesis no long abolish IL-2 induction (36). This suggests that inhibition can still occur after critical transcription factor synthesis, possibly by direct interactions between the GR and these factors and/or DNA. The results shown in Fig. 1 *B*, lanes *8–10*, using the NF-AT/Tag mice are quite different; while IL-2 expression is again inhibited by 50%, the transgene is unaffected. This indicates that NF-AT is likely not the target for inhibition in splenocytes.

The above results indicate that a fragment of the IL-2 gene encompassing only 600 bp of upstream sequences can, when stably integrated into the genome, behave identically to the endogenous IL-2 gene. We next tested whether a smaller fragment of IL-2, −325 to +47, previously shown to contain the IL-2 tissue- and activation-specific enhancer (37, 38), would confer negative regulation by dexamethasone. Fig. 1 *C* shows that induction of pIL-2CAT transfected into jurkat cells is almost completely abolished by dexamethasone treatment, while a construct with three copies of NF-AT directing expression of CAT appears relatively insensitive to hormone. In addition, the IL-2 enhancer construct in this transient expression assay is inhibited to a greater extent than that found for endogenous IL-2 or the IL-2/*c-myc* transgene.

Structural Requirements of the GR for Negative Regulation. Domains of the GR important for transcriptional activation have been well characterized (24, 39). We wanted first to test if the structural requirements of the GR needed for IL-2 repression were similar to those required for transcriptional activation. Our clone of jurkat cells is insensitive to glucocorticoids, as Western blotting reveals little if any GR protein, and a transfected glucocorticoid responsive reporter construct, MMTVCAT, is not inducible upon dexamethasone treatment in these cells (data not shown). Cotransfection of the mouse GR expression vector pSV2Wrec results in an ∼18-fold induction of MMTVCAT in jurkat cells (Fig. 2). When cotransfected with pIL-2CAT (Figs. 2 and 1 *c*), wild-type GR is able to inhibit >80% of the normal induction of the IL-2 enhancer, indicating that a functional receptor protein is necessary for repression. The control construct,



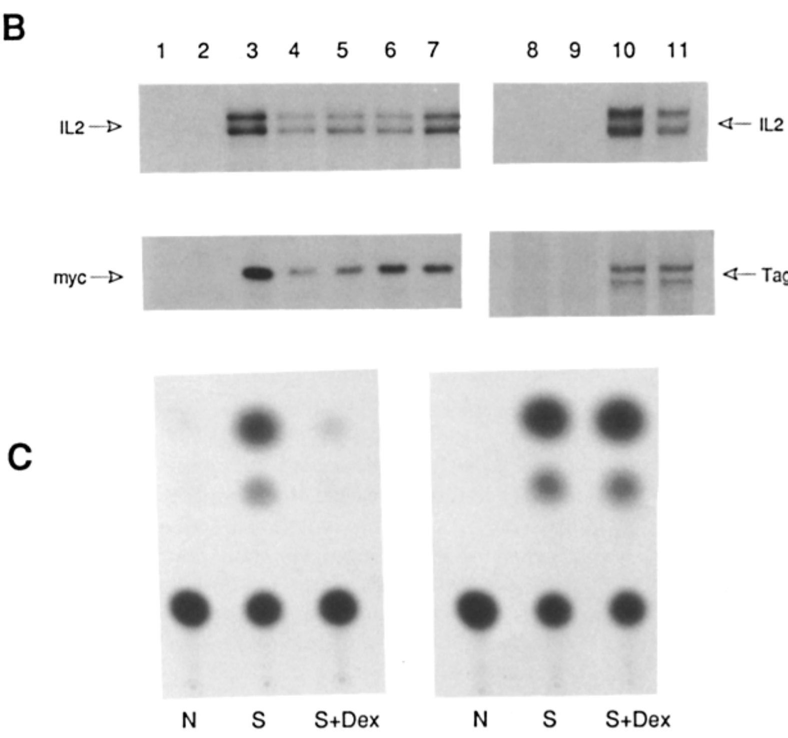


Figure 1. Repression of endogenous IL-2 and IL-2 enhancer or NF-AT-driven reporter genes in transgenic mice and jurkat cells. (*A*) Constructs used to generate transgenic mice. Top shows c-*myc* placed downstream of a 600-bp fragment of the human IL-2 gene containing the whole enhancer and promoter. Bottom shows SV40 T antigen driven from the minimal IL-2 promoter with three copies of the binding site for NF-AT. (*B*) Ribonuclease protection to detect endogenous murine IL-2 (*top*) and the transgenes IL-2/c-*myc* (lanes *1–7*, *bottom*) and NF-AT/T-antigen (lanes *8–11*, *bottom*). Isolated splenocytes were either not stimulated (lanes *2* and *9*) or stimulated for 3 h with PMA and ionomycin in the absence (lanes *3* and *10*) or the presence of dexamethasone added at the following times relative to stimulus addition. Lane *4*, −1 h; lanes *5* and *11*, 0 h; lane *6*, +1 h, and lane *7*, +2 h. Lanes *1* and *8* are tRNA-containing control samples. (*C*) GR-deficient jurkat cells were transiently cotransfected with 10 μg of pSV2Wrec and either 10 μg of pIL-2CAT or 10 μg of NF-AT p22-6 by electroporation. Cells were treated for 8 h with either solvent alone (nonstimulated, *N*), PMA plus ionomycin (stimulated, *S*), or PMA plus ionomycin plus dexamethasone (*S + Dex*). CAT activity was then assayed in whole cell extracts.

pSV2neo, does not confer repression of pIL-2CAT. An NH_2-terminally truncated GR construct, Δ395, has little activity on MMTVCAT (Fig. 2) (24), however, it retains almost full capacity to repress pIL-2CAT. These results indicate that the NH_2-terminal half of the GR, while containing a transcriptional activation domain, is not necessary for negative regulation. This is analogous to previous findings with the chorionic gonadotropin α subunit (HCG) gene (40) and the collagenase gene (14).

The above result indicates that structural requirements of the GR needed for repression and activation may differ significantly. A synthetic GR, where arginine 484 is mutated to histidine, is a very poor repressor (Fig. 2), indicating that an intact DNA binding domain is required for repression. Since this mutant is also defective in nuclear translocation (23, 41), this result does not necessarily indicate that DNA binding is required for repression. To further address this question, we tested three more constructs, each with a fully functional DNA binding domain, but whose target hormone-responsive element specificity had been changed by DNA binding domain swaps with either thyroid receptor or estrogen receptor (GE), or by two amino acid changes in the first zinc finger that changes the hormone-responsive element to that of the estrogen receptor (ΔEG). These constructs activate transcription from an estrogen-responsive element (GE, ΔEG) (25) or thyroid-responsive element (TR) (L. Hinck, personal communication), but not from a glucocorticoid-responsive element, and therefore have different DNA binding specificities from that of the GR. We found that these hybrid receptors also functioned as efficient repressors of IL-2 enhancer activity (Fig. 2). The fact that the construct GE represses only 40% as efficiently as wild-type GR is consistent with its 40% efficiency as compared with ΔEG for activation of an ERECAT construct (25). These results do not favor a mechanism of repression involving direct contact of GR with DNA, as is the case for positive hormone-responsive

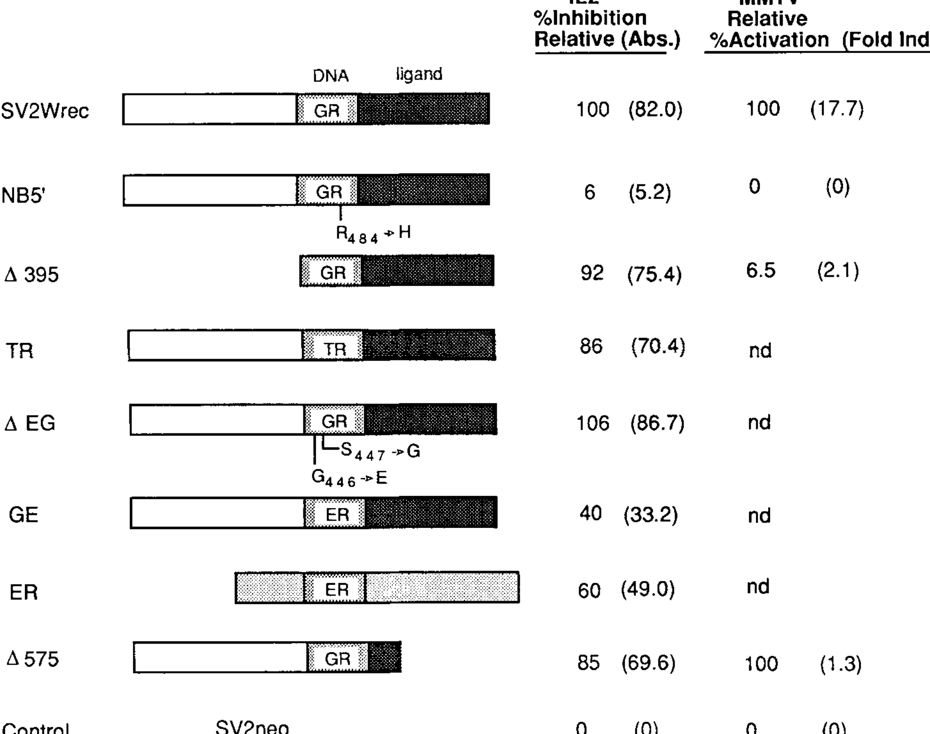


Figure 2. Deletion and mutation analysis of the GR. Various GR expression constructs, wild-type ER, or control SV2neo depicted on the left were cotransfected into GR-deficient jurkat cells with pIL-2CAT as described in Fig. 1 or with the glucocorticoid-responsive reporter construct MMTVCAT. Cells transfected with pIL-2CAT were treated with PMA plus ionomycin in the presence or absence of dexamethasone as described in Fig. 1 or with 10^{-7} M estradiol (for ER). MMTVCAT-transfected cells were treated with or without dexamethasone for 8 h before harvest for CAT assays. The results for pIL-2CAT are shown as absolute (*Abs.*) inhibition of PMA plus ionomycin-induced CAT activity or relative to the inhibition produced by the wild-type (*SV2Wrec*) construct. The inhibition by Δ575 was constitutive and was calculated using RSVCAT as an internal control and pIL-2ZH as the reporter. The results for MMTVCAT are shown as fold induction of CAT activity or relative to the wild-type construct. Not determined (*nd*). The results represent the mean of two to six independent experiments.

elements. We next investigated the function of the hormone binding domain in repression of the IL-2 enhancer by both changing and deletion of this region. Since a hybrid GR containing the ER DNA binding domain is a functional repressor, and the NH_2-terminal half of the receptor is not necessary, we reasoned that the wild-type ER might also be an effective repressor when suitably activated. Fig. 2 shows that the ER does function as a repressor, although only 60% as efficiently as the GR. This repression was not seen when cells transfected with the ER were treated with dexamethasone (data not shown). Deletion of a sufficient proportion of the hormone binding domain to produce the constitutive transcriptional activator, Δ575 (Fig. 2) (24), also results in a constitutive repressor of the IL-2 enhancer, which is only slightly less efficient that the wild-type GR. These results indicate that the specificity of the hormone binding domain is not critical and that, to a large extent, this domain is not necessary for repression.

Repression of IL-2 Occurs through More Than One Transcription Factor Binding Site. The above experiments demonstrated that an IL-2 enhancer containing only 325 bp of upstream sequence is able to mediate glucocorticoid repression. Additionally, GR structural requirements for IL-2 repression are similar to those found for repression of Jun/AP-1 activity (14, 40). In fact, at least two AP-1-like sequences have been identified within the IL-2 enhancer at approximately −147 and −181 (42), the former of which lies within a functionally important region (28). To determine the site(s) mediating repression, a series of deletion mutants of the IL-2 enhancer was tested for repression by a cotransfected GR in jurkat cells. Since the IL-2 enhancer is virtually silent in the absence of stimulation (36) (Fig. 1), and glucocorticoids inhibit activation of the enhancer in response to stimulatory signals, we focused our studies on previously identified functional regions of the enhancer. Deletion of the NF-AT binding site in LS5 leads to a construct that is highly repressible (Fig. 3 *A*). This is consistent with data presented in Figs. 1 *C* and 3 *B*, showing little repression through multimerized NF-AT sites. Deletion of a second purine-rich sequence similar to the NF-AT site also has no effect on repression (LS1 in Fig. 3 *A*). Two more deletions (LS29, which deletes the NFIL-2C [NFκB like] sequence and an AP-1-like sequence, and LS20, which deletes the NFIL2B [also AP-1 like] sequence) are repressed to an extent similar to that of the wild-type enhancer (Fig. 3 *A*). These results indicate that none of these sites alone are responsible for GR repression of the IL-2 enhancer. Deletion of NFIL-2A (Oct1/OAP) site leaves an enhancer with so little ability to be activated (28) that repression could not be tested in this construct. Duplication of the proximal half of the enhancer containing the NFIL-2A and NFIL-2B sites as in DSCAT (Fig. 3 *A*) results in an inducible construct that retains the wild-type ability to be repressed.

We then used a series of constructs where multimerized individual sites from the IL-2 enhancer are linked to a minimal IL-2 promoter. The results of these experiments using identical constructs driving either CAT or *lacZ* are presented in Fig. 3 *B*. While a construct driven by three NFκB binding sites shows no repression, two sites, NFIL-2A (Oct p22-6 and OctZH) and NFIL-2B (NFIL-2BZH and APCAT), show >50% repression. It is likely that the deletion mutant LS20, which lacks the NFIL2B site (see above), remains repressible because it still contains an intact NFIL-2A site. These results

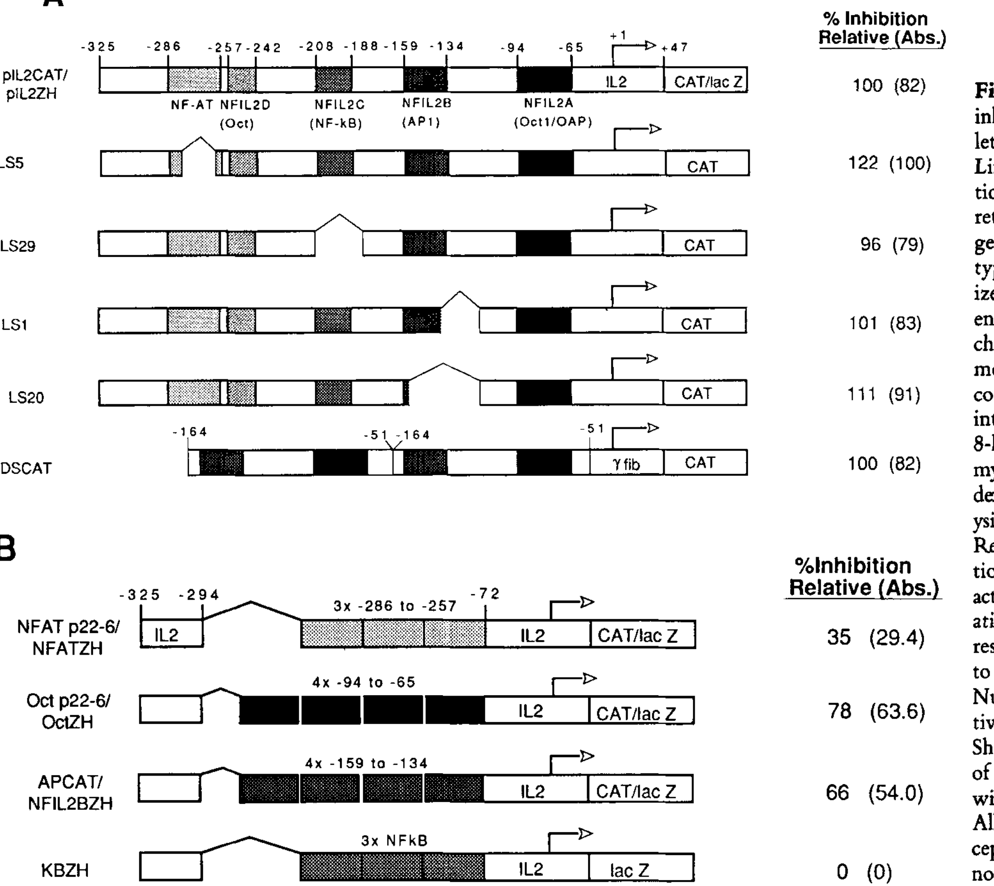


Figure 3. Mapping the site of GR inhibition in the IL-2 enhancer. (*A*) Deletion analysis of the IL-2 enhancer. Linker-scanner generated internal deletions of the IL-2 enhancer, with each retaining significant inducibility but in general somewhat lower than the wild-type enhancer (28); or (*B*) multimerized factor binding sites from the IL-2 enhancer or NFκB site from κ light chain enhancer linked to the *IL-2* promoter and either CAT or *lacZ* were cotransfected with 10 μg of pSV2Wrec into jurkat cells as described in Fig. 1. 8-h stimulations with PMA plus ionomycin in the presence or absence of dexamethasone were followed by analysis of CAT or B-galactosidase activity. Results are presented as percent inhibition of inducible CAT/B-galactosidase activity in absolute (*Abs.*) terms and relative to the complete enhancer. The results represent the mean of from two to seven independent experiments. Numbers above the constructs are relative to the start of IL-2 transcription. Shaded boxes represent functional areas of the enhancer, named below the box, with factor binding sites in parenthesis. All constructs use the IL-2 promoter except DS CAT, which uses the γ fibrinogen promoter.

raise the intriguing possibility that like activation of the IL-2 enhancer, which requires multiple transcription factor sites for maximal activity, maximal repression may also involve interaction of the GR with more than a single factor and/or binding site.

NFIL-2A and NFIL-2B Sites Bind Similar AP-1-related Factors. As presented above, glucocorticoid repression occurs through both the NFIL-2A and NFIL-2B sites (Fig. 3 *B*). Because the NFIL-2B site contains an AP-1-like sequence, we investigated whether the NFIL-2A site also might contain a similar sequence. Comparison of the NFIL-2A and NFIL-2B sequences with a consensus AP-1 sequence (Fig. 4 *A*) reveals the presence of the 6/7-bp match in the NFIL-2B sequence and a 5/7-bp match in the NFIL-2A sequence. This region of the NFIL-2A sequence lies immediately upstream from an octamer site and binds a recently discovered inducible factor, octamer-associated protein (OAP) (43). Coocupancy by Oct1 and OAP of the NFIL-2A oligonucleotide can be observed in a gel mobility shift. To test the possibility that OAP may be similar to the factor that binds to NFIL-2B site, we ran a series of electrophoretic mobility shift assays using nuclear extracts from stimulated jurkat cells. The three oligonucleotides, OAP, NFIL-2B, and a consensus AP-1 binding site, give rise to retarded bands with similar mobilities (Fig. 4 *B*). In addition, both the OAP and NFIL-2B sites cross-compete with the consensus AP-1 site, while the unrelated sequence, NF-AT, does not compete. Fig. 4 *B*, lane *17*, shows that the AP-1 site specifically competes the OAP-containing band and not the band containing Oct1 only. These results suggest that OAP and the factor that binds to the NFIL-2B site are highly related or even identical and have binding specificities similar to AP-1. As negative regulation of the IL-2 enhancer appears to occur through interactions with these factors, a family of AP-1-related proteins are likely capable of being negatively regulated by glucocorticoids.

Critical Factors for IL-2 Enhancer Function Are Unaltered by Glucocorticoid Treatment. The results above led us to predict that glucocorticoid treatment of sensitive (GR-containing) T lymphocytes during mitogenic stimulation would not alter the intrinsic properties or quantities of various factors involved in IL-2 transcription. To test this hypothesis we used the glucocorticoid-sensitive murine T cell line, LBRM, which like jurkat cells produces IL-2 when mitogenically stimulated. LBRM cells were stimulated in the presence or absence of dexamethasone and nuclear extracts tested for factor binding by gel shift. The constitutive factor Oct1 and the inducible factors binding to an AP-1, an NF-AT, and the OAP sites are all unaffected either quantitatively or qualitatively by dexamethasone treatment in vivo (Fig. 5). These results indicate that glucocorticoid treatment does not act indirectly by in-

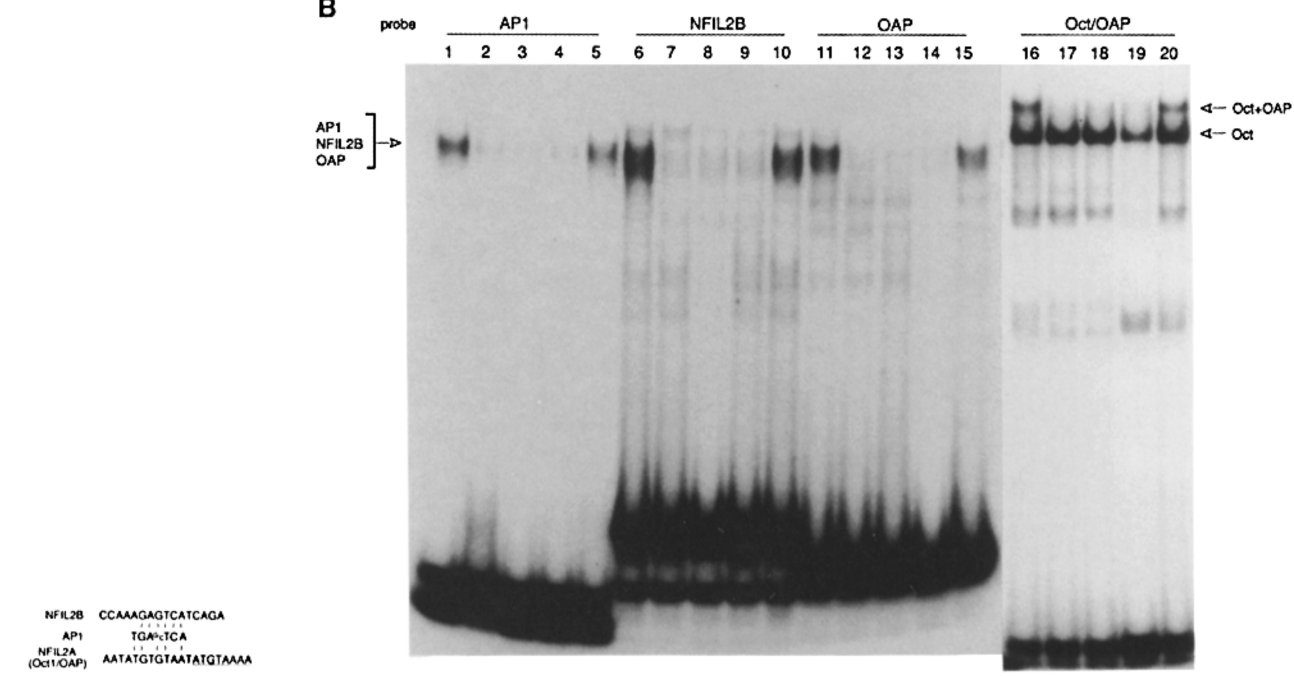


Figure 4. Gel mobility shift analysis using AP-related sequences within the IL-2 enhancer. (*A*) Sequence comparison of the AP1 like sequences within the NFIL-2B and NFIL-2A sites with a consensus AP-1 site. The actual oligonucleotides containing these sequences and used for gel shifts are described in Material and Methods. (*B*) Gel mobility shifts using labeled oligonucleotide probes as shown and nuclear extracts from PMA plus ionomycin-stimulated jurkat cells. Competitor oligonucleotides are none (lanes *1*, *6*, *11*, and *16*), AP-1 (lanes *2*, *7*, *12*, and *17*), NFIL-2B (lanes *3*, *8*, *13*, and *18*), OAP (lanes *4*, *9*, *14*, and *19*), and NF-AT (lanes *5*, *10*, *15*, and *20*).

hibiting synthesis of these transcription factors. Direct interactions between the GR and other transcription factors are not assayable in this experiment, as the method used to prepare the nuclear extracts does not recover GR and, therefore, the extracts used are essentially devoid of GR by either gel shift or Western analysis (data not shown).

Discussion

We have presented a series of studies aimed at defining the mechanism by which the GR inhibits IL-2 transcription. Our initial findings using transgenic mice show that a stably integrated construct containing 600 bp of IL-2 upstream sequence is repressible by glucocorticoid treatment to the same extent as the endogenous murine IL-2 gene (Fig. 1). This confirms results presented using transient transfections (22) and demonstrates that the IL-2 enhancer mediates repression not only in a lymphoblastoid T cell line but also in splenocytes. Importantly, the results indicate that transient transfection methods are a valid approach for further analysis of glucocorticoid inhibition of IL-2 gene expression. We show, further, that the minimal IL-2 enhancer/promoter, containing only 325 bp of upstream sequence, is capable of glucocorticoid repression. The comparison between the whole IL-2 enhancer and the three NF-AT sites in Fig. 1 *B* has the caveat that the reporter transcripts are not the same. It is therefore possible that the differences seen reflect transgene expression differences rather than enhancer construct differences. Our results using the CAT reporter gene in transient transfections, however, corroborate these results showing a large difference between the negative regulation of the intact enhancer and the multimerized NF-AT sites.

While the inhibition of IL-2 message accumulation in splenocytes is only slightly over 50%, inhibition of reporter gene activity in transient assays is far greater (Figs. 1–3). This suggests the possibility of a translational component to the reporter gene regulation, however, this is unlikely since the construct containing NFκB sites shows no inhibition (Fig. 3 *B*). In addition, the lack of hormone-mediated inhibition of the NFκB construct argues against an effect on reporter gene mRNA stability. The likely explanation for the more complete inhibition seen in jurkat cells as compared with splenocytes may to be a limitation in the number of receptors per cell. We have found that expression of wild-type GR from a more active promoter in jurkat cells leads to even greater inhibition (data not shown). Likewise, Vacca et al. (22) found that inhibition of IL-2-driven CAT expression could be increased even in steroid-sensitive jurkat cells by cotransfection of a GR expression vector. However, it is still possible that mechanisms other than repression of transcription contribute to glucocorticoid-mediated downregulation of IL-2 expression. IL-2 production from rat spleen cells or human PBLs can be almost completely inhibited by 24 or 48 h dexamethasone treatment (20) and, therefore, glucocorticoids may also

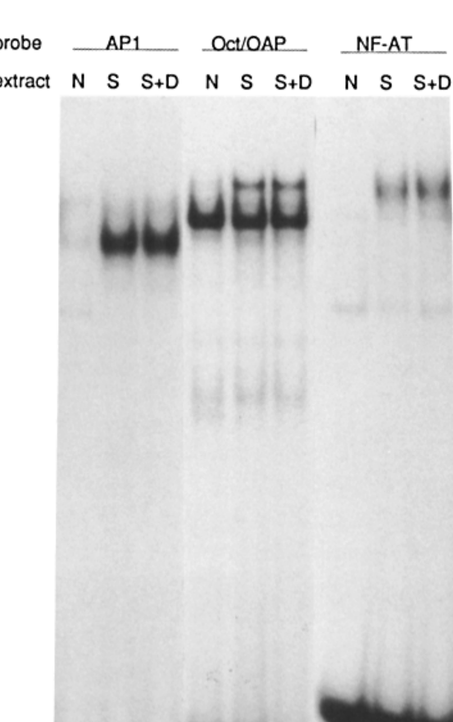


Figure 5. Gel mobility shifts using nuclear extracts from dexamethasone-sensitive LBRM cells. LBRM cells were not stimulated (*N*) or stimulated for 3 h with PMA plus ionomycin in the presence (*S* + D) or absence (*S*) of dexamethasone and nuclear extracts prepared. Gel mobility shifts were done using the indicated probes.

have an effect on IL-2 mRNA stability, as was found for the IL-1β gene (5), IL-2 mRNA translation, or IL-2 protein stability.

A number of our results suggest strongly that glucocorticoid inhibition of IL-2 transcription is not mediated by indirect pathways. First, dexamethasone treatment of GR containing LBRM cells does not inhibit production of critical transcription factors involved in IL-2 enhancer function. Second, inhibition of IL-2 message accumulation can still be observed when hormone is added after the time when these same factors and IL-2 message become apparent. Third, the DNA binding specificity of the receptor can be altered to that of the estrogen or thyroid receptors without loss of repressor function, indicating that induction of a second gene, presumably through the interaction of the receptor with a GRE, cannot be occurring.

Deletion and mutation analysis of the GR protein reveals that the structural requirements for activation and repression are different. The NH_2-terminal half, which contains a transcriptional activation domain, is dispensible in repression. Similar results have been found for GR repression of the HCG (40) and collagenase (14) genes. As with transcriptional activation, an intact DNA binding domain is essential for repression, as a single point mutation in this domain renders the receptor essentially inactive as a repressor. However, this does not mean that binding to DNA is required for repression, but only indicates that some critical function of the DNA binding domain can be lost by mutation. Indeed, we have been unable to show binding of a bacterially expressed GR DNA binding domain, which binds well to a consensus GRE in vitro, to the OAP or NFIL-2B sites identified as functionally important regions in glucocorticoid repression (data not shown). Our observation that receptors with the DNA binding specificity of the ER or TR both work as efficient repressors supports the view that DNA binding is not required. We therefore favor a mechanism by which the GR interacts directly with transcription factors. Likely, the overall structure of the zinc finger motifs within the required DNA binding domain needs to be maintained for receptor interactions with other factors. Our data are consistent with previous observations indicating that the DNA binding domain is necessary for repression of the collagenase (14) and HCG (40) genes. We have shown that the hormone binding domain of the receptor need not be that of the GR for IL-2 repression. In fact, the wild-type ER functions as a reasonably efficient repressor in response to estradiol. Previous studies in other repression systems (14, 40) have shown that the GR hormone binding domain can be replaced functionally by that of the retinoic acid or mineralocorticoid receptors or even β-galactosidase. Thus, IL-2 repression by the GR appears similar to these examples. Deletion of a large proportion of the hormone binding domain gives rise to a constitutive repressor of the IL-2 enhancer, which is nearly as active as the wild-type receptor. This GR construct is a more efficient repressor than similar constructs used in other systems (14, 40), likely because it leaves a greater proportion of the COOH-terminal half of the receptor intact. Alternatively, the requirements for efficient repression of the OAP factor differ somewhat from those of AP-1.

Recently, it has been demonstrated that functional antagonism and protein-protein interactions occur between the transcription factor AP-1 (specifically cJun) and the GR (12–14). Previous studies (42) have identified two potential AP-1 binding sites within the IL-2 enhancer, one of which lies within the functionally important region, NFIL-2B (28). We have identified the NFIL-2B site as one region that mediates repression by glucocorticoids, and it is therefore possible that IL-2 repression by glucocorticoids occurs by interactions between the GR and cJun. Interestingly, however, previous studies (44) have shown that the core AP-1-like sequence of the NFIL-2B site, AGAGTCA, does not bind cJun/Fos heterodimers. It therefore seems unlikely that the factor (likely OAP) that binds to the NFIL-2B site contains cJun. We have shown that the NFIL-2A site also mediates repression and contains a sequence similar to an AP-1 binding motif. OAP, which mediates inducibility through the NFIL-2A site, is able to bind to the NFIL-2B site (unlike cJun) and to a consensus AP-1 site (Fig. 4). OAP is thus a candidate for a factor, distinct from cJun, that is functionally inactivated by the GR during glucocorticoid-mediated repression of IL-2 transcription.

The precedent for a family of AP-1 factors exists with the identification of at least three members of the Jun family and

a number of Fos-related proteins (45–52). In spite of some apparent similarities between AP-1 and OAP, a clear functional distinction should also be emphasized. PMA treatment in the absence of ionomycin does not activate the NFIL-2A or NFIL-2B sites in vivo, while a construct containing a consensus AP-1 site is inducible with PMA alone (31). Activation of the NFIL-2A and NFIL-2B sites, but not the consensus AP-1 site, is sensitive to the immunosuppressive drug cyclosporin A (31). In addition, we have tested a number of antisera directed against cJun and Fos proteins and have seen no crossreactivity with the OAP factor (K. Ullman, J. Northrop, and G. Crabtree, unpublished results). We therefore propose that although OAP has a binding specificity somewhat similar to that of AP-1, it is functionally distinct from AP-1 and likely does not contain cJun. The identity of OAP is not known at this time, however, work in our laboratory is currently directed towards this goal. The GR is therefore capable of interacting with or inhibiting the activity of a variety of transcription factors.

In summary, we have studied the mechanism of glucocorticoid inhibition of IL-2 gene expression in T lymphocytes and have identified functionally important sequences within the enhancer as sites of repression. Nuclear factors that bind to these sites are related to but distinct from AP-1, but like AP-1, are targets for repression by the GR. These studies help explain the growth inhibitory and functional suppressive effects of glucocorticoid hormones on the immune system.

We are grateful to Mark Danielson and Lindsay Hinck for providing GR constructs, to M. Parker for the construct pJ3MOR, to Keith Yamamoto for the T7X556 protein (bacterially expressed GR DNA binding domain), to Tom Curran and M. Karin for antisera against Fos and cJun, to Cor Verweij and E. Lacy for the transgenic mice, to Katie Ullman for many helpful discussions, and to Jean Oberlindacher for help in preparing this manuscript.

This work was supported by grants from the National Institutes of Health (HL-33942 and CA-39612) and the Howard Hughes Medical Institute (to G. R. Crabtree), by NIH grant CA-42509 to Leonard A. Herzenberg, by U.S. Public Health Service postdoctoral fellowship in Immunology grant AI-07290 to J. P. Northrop, and by a grant from the Paolo Foundation to P. S. Mattila.

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Received for publication 30 August 1991 and in revised form 20 December 1991.

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