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Regulation of the Glucocorticoid Receptor Gene by the AP-1 Transcription Factor

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The glucocorticoid receptor (GR) is a ligand-activated nuclear transcription factor, and AP- 1 (Fos/Jun or Jun/Jun) is a transcription factor whose components are nuclear proteins encoded by c-*fos* and c-*jun* proto-oncogenes. Serum stimulation of serum-starved NIH 3T3 cells resulted in an approx 188-fold induction of c-*fos* mRNA at 30 min and an approximately ninefold induction of c-*jun* mRNA at 1 h, followed by an increase in GR mRNA levels at 3–12 hour (twofold). Sequential induction of cFos, cJun, and GR protein levels also occurred. Overexpression of the cFos protein in NIH 3T3 cells (NIH 3T3 [cFos 3] and NIH 3T3 [cFos 10]) caused an increase in the endogenous GR protein. Previous and present studies showed that a putative AP-1 site within the GR promoter binds AP-1 proteins (both Jun and Fos family members). To address the molecular mechanism involved in transcriptional activation of the GR gene, we investigated the relevance of AP-1 binding complexes in this activation and in overall regulation of GR gene transcription. Transient transfection with a full length GR promoter linked to a luciferase gene into both NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10) cells gave rise to an induction of luciferase activity. This induction was abolished following mutation or deletion of the GR AP-1 site from the promoter. These findings suggest that cFos is responsible for the induction of GR expression in serum-stimulated NIH 3T3 cells, and serum growth factors may stimulate GR transcription by a cFos-dependent mechanism at the putative AP-1 site. These studies support a role for the AP-1 transcription factor in regulating GR gene expression.

Key Words: Glucocorticoid receptor; AP-1; Fos; promoter.

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Introduction

Glucocorticoid hormones serve a variety of crucial functions in regulating development, differentiation, and homeostasis *(1,2)*. They exert their regulatory effects by binding to their intracellular receptor, the glucocorticoid receptor (GR), with high affinity and specificity. The GR is a ligand-dependent transcription factor that belongs to the nuclear hormone receptor family *(1,2)*. It mediates the biological effect of glucocorticoids by both positive and negative regulation of gene expression *(3–5)*. Previous studies showed that the GR is present in many cell types and tissues with different expression levels *(6,7)*. It was also suggested that the intracellular concentration of GR protein can control the magnitude of the cellular response *(8,9)*. Since the biological effects of glucocorticoids are dependent on the presence of functional receptors, elucidation of the mechanisms involved in GR gene expression is of particular importance to understanding how cellular GR levels are regulated.

The promoter regions of both the human *(10–13)* and mouse *(14)* GR genes have been characterized. Using a 75% or better identity to consensus sequences, many putative *cis*-acting elements were identified within the human GR promoter *(10)*. A more recent study revealed 11 protein binding sites in this promoter using in vitro DNase I footprinting *(13)*, and AP-2 and Sp1 transcription factors are found to bind to the GR promoter. An additional Sp1 site and four YY1 transcription factor binding sites have also recently been identified in the human GR promoter (Breslin and Vedeckis, submitted).

Among the putative *cis*-acting elements revealed by sequence analysis, a potential AP-1 site is located at –899 to –893 in the human GR promoter. This AP-1 site (TGACAACA) differs from the consensus AP-1 site ($TGA^{C}/_{G}TCA$) by only one nucleotide (underlined). We had previously shown that this GR AP-1 site can be bound by different AP-1 proteins (both Jun and Fos family members) in vitro *(15)*.

The present study was undertaken to define the role of the AP-1 protein in the in vivo regulation of the GR gene. We show that serum stimulation of NIH 3T3 cells causes a

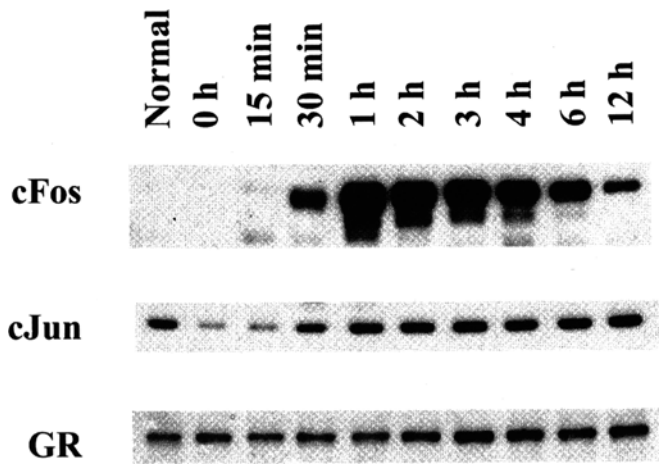


Fig. 1. Time-course of induction of the cFos, cJun, and GR proteins in serum-stimulated NIH 3T3 fibroblasts. Quiescent NIH 3T3 cells (48 h in 0.5% Colorado calf serum/DMEM) were stimulated with 20% serum for the indicated times. Whole-cell extracts were subjected to Western blot analysis using anti-cFos, anti-cJun, and anti-GR antibodies. Normal refers to an extract obtained from normal logarithmically growing NIH 3T3 cells.

sequential induction of the c-*fos*, c-*jun*, and GR genes. Overxpression of the cFos protein both induces endogenous GR protein levels and stimulates GR promoter activity. Supershift assays revealed binding of the Fos protein to the putative GR AP-1 site. Finally, deletion or mutation of this site abolished induction of the GR gene by the Fos protein, suggesting that serum induction of GR gene expression may be mediated by the AP-1 site within the GR promoter.

Results

Expression of the Endogenous GR Gene in NIH 3T3 Cells Is Stimulated by Serum

Both c-*fos* and c-*jun* are immediate-early genes. Their transcription is rapidly induced by various extracellular stimuli, such as growth factors and cytokines present in serum (reviewed in *16*). When quiescent fibroblasts are exposed to whole serum, both c-*fos* and c-*jun* genes are rapidly stimulated *(17–18)*. To determine if the elevated AP-1 activity in response to serum stimulation can induce the expression of the GR gene, we studied the GR protein and mRNA levels in serum-stimulated NIH 3T3 cells. The cells were starved with medium containing 0.5% serum for 48 h and then treated with 20% serum for various times. The protein samples were analyzed by Western blot analysis, and the mRNA samples were subjected to a ribonuclease protection assay (RPA).

Sequential induction of the cFos, cJun, and GR proteins was seen after serum stimulation (Fig. 1). The cFos protein levels in normally cultured or quiescent cells were very low, almost undetectable. The cFos protein appeared rapidly within 15–30 min after the cells were exposed to serum. It fell rapidly after reaching a peak level at 1 h. Subsequent to cFos induction, the cJun protein level increased between 30 min and 1 h upon serum stimulation. Finally, induction of the GR protein occurred at 3 h. Similar to the cJun protein, GR protein levels plateaued after being induced. These increases in protein levels were accompanied by similar changes in the mRNA, although the induction of the c-*fos* and c-*jun* mRNA was transient and reached a maximal level within 30 min and 1 h after serum stimulation, respectively (Fig. 2). The GR mRNA level increased within 30 min after serum stimulation and remained substantially elevated for at least 12 h (Fig. 2). The fold induction of the GR protein and mRNA levels by serum was 2.5- to 3-fold of those in the control cells (Fig. 3). These results show a temporal pattern for cFos, cJun, and GR induction, and they suggest that the expression of the GR gene may be induced by AP-1 proteins, either cFos or cJun, or both.

The cFos Protein is Capable of Inducing the Expression of the GR Gene

To determine specifically if the cFos protein is responsible for the stimulation of GR gene expression, NIH 3T3 cells were stably transfected with a cFos expression vector, CMV-fos. Two of the positive clones, NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10), showed about a twofold increase in cFos protein expression compared to untransfected cells. In these two clones, endogenous GR protein levels are also about twofold higher than those in the untransfected cells (Fig. 4). These results indicate that overexpression of the cFos protein can induce endogenous GR gene expression.

The Putative AP-1 Site Within the GR Promoter is Essential for the Induction of the GR Gene by the cFos Protein

To identify the regulatory elements that are responsible for the cFos-mediated induction of GR gene expression, we transiently transfected NIH 3T3, NIH 3T3 (cFos 3), and NIH 3T3 (cFos 10) cells with human GR promoter deletion mutants fused to a luciferase reporter gene (LUC) (Fig. 5). These GR promoter/LUC constructs contain variable lengths of the GR promoter (-2738 to -890 bp). The –2738 and –1046 bp constructs, which contain the putative AP-1 site (–893 to –899), exhibited two- to approximately three-fold induction of GR promoter activity in the NIH 3T3 (cFos3) and NIH 3T3 (cFos 10) cells, which express two-fold more cFos than untransfected cells. By contrast, removal of the region between -1046 and -890 bp abolished this induction, suggesting that a sequence between -1046 and -890, probably the putative AP-1 site (-893 to -899), is involved in cFos regulation of the hGR gene. To elucidate the role of the putative AP-1 site in regulating GR gene expression by the cFos protein, this site was mutated or deleted from the –2738 bp promoter context using in vitro site-directed mutagenesis. Transfection data showed that mutation or deletion of this site did not affect the basal promoter activity in NIH 3T3 cells, but it reduced the Fos-induced luciferase activity by about 30–40% in the NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10) cells. There is an

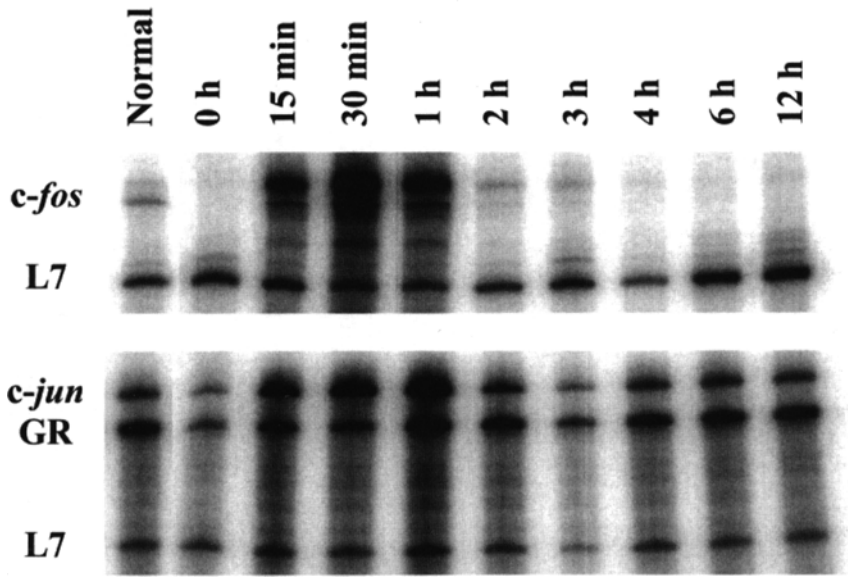
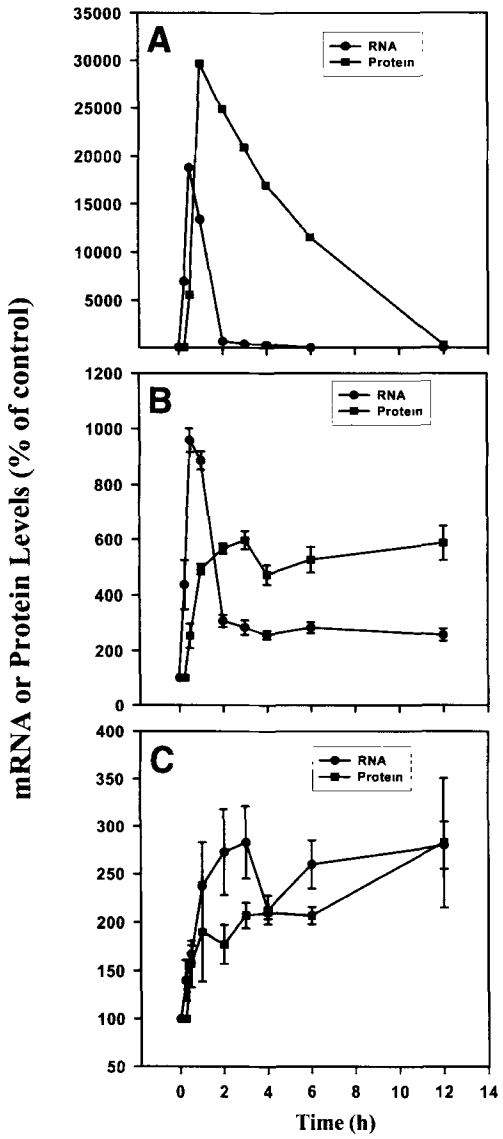


Fig. 2. Expression of the c-*fos*, c-*jun*, and GR genes on serum stimulation. Total RNA was isolated from the same flask of cells treated as in the legend of Fig. 1. The RNA samples were subjected to a ribonuclease protection assay (RPA). L7 represents mouse L7 ribosomal protein mRNA, which is used as an internal control.



apparent increase in promoter activity in the cFos-overexpressing cells (NIH 3T3 [cFos3] and NIH 3T3 [cFos10]) even when the AP-1 site is mutated or deleted. We are unsure of the reason for this, but it may result from a secondary effect (for example, overexpression of cFos may stimulate the expression of a different transcription factor that acts elsewhere in the GR promoter), or the "promoterless" plasmid backbone may have a cryptic sequence that responds to the increased cFos levels. However, it is obvious that cFos protein induced the promoter activity to a much greater degree when the GR AP-1 site is present. Thus, it seems clear that this additional increase in promoter activity in the cFos-overexpressing cells can be attributed to the AP-1 site in the GR promoter. Taken together, these results indicate that the putative AP-1 site is functional, and that the cFos protein stimulates GR transcription, at least in part, through this element.

The Putative GR AP-1 Site Interacts Specifically with the Fos Proteins

Electropheretic mobility shift assays (EMSAs) were performed to confirm the binding of the AP-1 proteins to the putative GR AP-1 site. When the NIH 3T3 nuclear

Fig. 3. Relative cFos, cJun, and GR protein and mRNA levels in serum-stimulated NIH 3T3 cells. The Western blots (Fig. 1) were densitometrically scanned and the data plotted as percent of the signal obtained at 15 min of serum stimulation. Following RPA (Fig. 2), the PhosphoImager data were analyzed using ImageQuant software (Molecular Dynamics). The signals for mouse L7 ribosomal protein mRNA obtained at each time-point were used to normalize the c-*fos*, c-*jun*, and the GR signals. Each data point was represented as percent of the signal obtained at 0 min of serum stimulation. Results are presented as the mean ± SEM of at least 3 independent experiments. **(A)** c-*fos*. **(B)** c-*jun*. **(C)** GR.

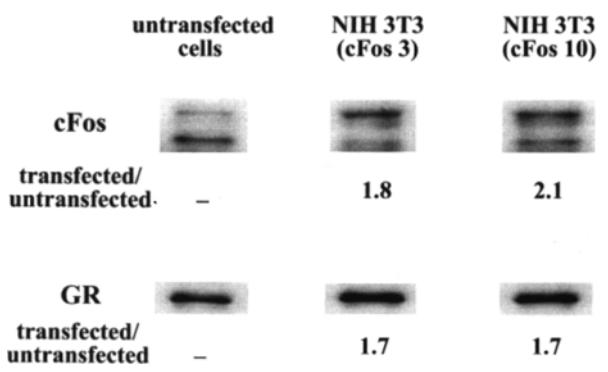


Fig. 4. Overexpression of the cFos protein-induced endogenous GR protein levels. A cFos expression plasmid was stably transfected into NIH 3T3 cells. Whole-cell extracts from two clones NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10) were subjected to Western blot analysis. The Western blots were densitometrically scanned, and the data presented as fold induction of the cFos or GR protein in transfected cells over untransfected cells.

extract was incubated with the wild-type GR AP-1 site, two protein–DNA complexes were observed (Fig. 6A). The lower complex appeared to be nonspecific. An anti-Fos antibody supershifted 100% of the upper complex, indicating that the GR AP-1 site was mainly bound by heterodimers of Jun and Fos family members. Surprisingly, the mutated GR AP-1 site also formed two protein–DNA complexes, both of which displayed different migration compared to the wild-type GR AP-1 site. Neither of these complexes was supershifted by the Fos antibodies, showing that other nuclear proteins interacted with the oligonucleotide containing the mutant GR AP-1 site. Under identical experimental conditions, the nuclear extracts from serum-stimulated NIH 3T3 and NIH 3T3 (cFos 3) cells exhibited similar patterns of binding. However, more Fos proteins were bound to the GR AP-1 site (upper band) (Fig. 6A). The fact that neither the lower band (wild-type oligo) nor the two new bands formed with the mutant oligo exhibited more complex formation after serum stimulation suggests again that non-AP-1 proteins are in these complexes.

To determine if the new DNA–protein complexes specifically required the mutated AP-1 site to form, we incubated the NIH 3T3 nuclear extracts with various oligonucleotides. The wild-type AP-1 oligo formed a complex that we had shown to contain AP-1 proteins (Fig. 6B, lane 1). A slower-migrating, unknown protein–DNA complex was again seen with the mutated GR AP-1 oligo containing both flanking regions (Fig. 6B, lane 2). By contrast, an oligo in which the AP-1 site was deleted and that contained both of the flanking regions was not bound by this protein (Fig. 6B, lane 3). Additional studies indicated that both flanking sequences plus the mutated AP-1 site were required to form this slower-migrating complex (data not shown). Therefore, DNA binding of the unknown protein occurs specifically at the mutated AP-1 site and requires both flanking sequences. Although the nature of this unknown protein remains unclear, its binding to the "AP-1 mutation," if it occurs in the intact cell, has no effect on GR promoter activity in the cell (compare "AP-1 mutation" and "AP-1 deletion" results in Fig. 5).

Discussion

Despite the fact that the GR is widely expressed in nearly all cell types and it plays an essential role in cell metabolism and growth, the regulation of GR gene expression remains unclear. In the present studies, we discovered that expression of the GR gene was upregulated by serum growth factors. This upregulation may be owing to the elevated AP-1 proteins, most probably the cFos protein. Examination of the sequence contained within the GR promoter region revealed the presence of a putative AP-1 site *(10)*. Deletion and mutation analysis of this site showed that it may mediate an increase in the GR promoter activity by the cFos protein.

The GR AP-1 site (TGACACA) differs from the consensus AP-1 site (TGA$^{C}/_{G}$TCA) by a single nucleotide substitution (underlined). An identical site was found to be present in the backbone of the pUC19 plasmid *(19)*. When linked to a CAT reporter gene, it is capable of stimulating transcription *(19)*. A more recent study indicated that hGR promoter sequences, which contain the putative AP-1 site, function as enhancer sequences. Warriar and colleagues *(20)* proposed that these sequences are responsible for high-level expression of GR in HeLa and placenta cells. In our studies, we demonstrated that this putative AP-1 site did not appear to be required for the basal activity of the GR promoter in logarithmically growing NIH 3T3 cells. However, this AP-1 site mediates induction of the GR promoter activity when the cFos protein is overexpressed in these cells (Fig. 5). Therefore, the enhancer function of this AP-1 site is cell-type-specific and depends on the level of intracellular components; this may partially account for the diverse expression levels of the GR gene in various tissues and cells.

A comparison of the mouse and human GR promoter sequences revealed that the AP-1 site at -893 to -899 in the human promoter is not conserved in the mouse GR promoter (Fig. 7; see *13*). This was surprising, since the original-serum stimulation and cFos-overexpression studies demonstrated an induction of endogenous GR gene expression in mouse NIH 3T3 cells (Figs. 1–4). However, a closer examination of the mouse GR promoter showed that an identical, nonconsensus (TGACACA) AP-1 site is found about 40 bp downstream from the corresponding location of the human AP-1 site (Fig. 7). The human GR promoter does not contain an AP-1 site at the location in which it is found in the mouse GR promoter. The fact that the same nonconsensus AP-1 site, although not precisely conserved with respect to promoter location, is found in the same

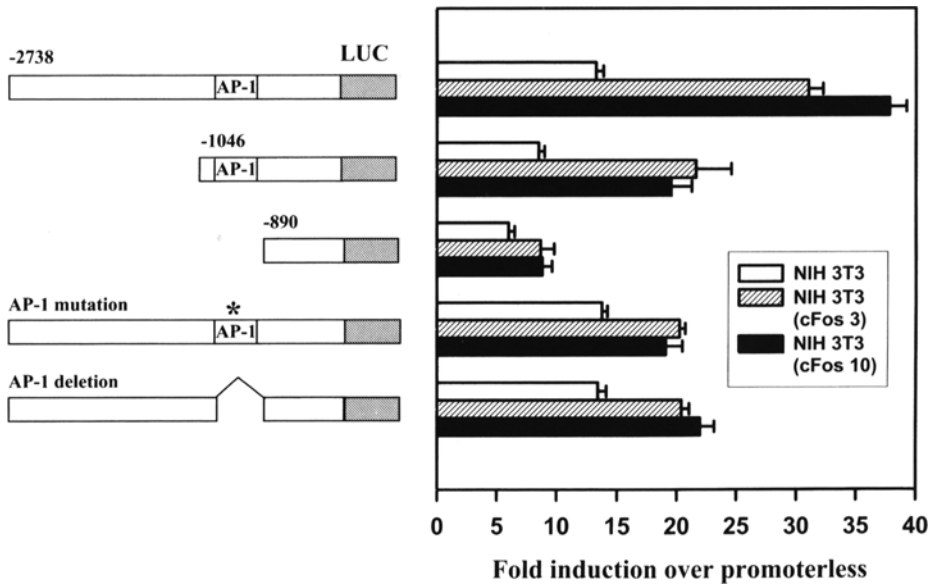


Fig. 5. GR promoter activity is stimulated by the cFos protein via the GR AP-1 site. The schematic diagram represents a series of GR promoter deletion mutant-luciferase gene constructs containing variable 5'-ends (from -2738 to -890), or a full-length promoter containing mutation or deletion of the putative AP-1 site. Each construct was transiently transfected into NIH 3T3, NIH 3T3 (cFos 3), or NIH 3T3 (cFos 10) cells for 48 h, followed by a luciferase assay. Promoter activity is normalized for transfection efficiency by dividing the luciferase activity by the β-galactosidase activity of a cotransfected pCH110 reporter plasmid, and it is expressed relative to the promoterless pGL3-Basic vector.

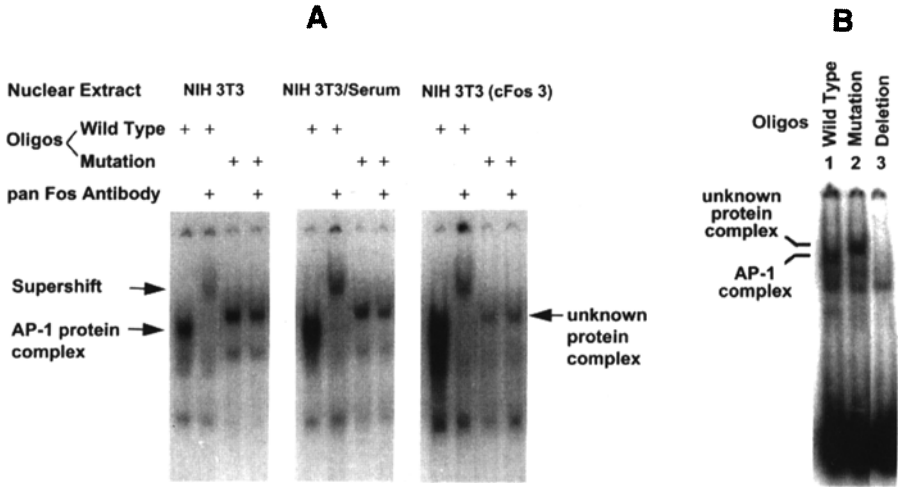


Fig. 6. Electrophoretic mobility shift assays (EMSAs) of the human GR AP-1 site. EMSAs were performed with 10–15 μg of the nuclear extracts of NIH 3T3, serum-stimulated NIH 3T3, and NIH 3T3 (cFos 3) cells. **(A)** [^{32}P]-labeled oligonucleotides for the wild-type or mutated human GR promoter AP-1 site were used. The AP-1 protein complexes were supershifted with 2 μg of pan Fos antibodies, which recognizes all the Fos family members. The "unknown protein complex," which migrated somewhat slower than the "AP-1 protein complex" was only seen using the mutated AP-1 site oligonucleotide. **(B)** EMSAs were performed with NIH 3T3 nuclear extracts and [^{32}P]-labeled oligonucleotides containing the wild-type AP-1 site (lane 1) and the mutated AP-1 site (lane 2), or an oligonucleotide in which the AP-1 site was deleted, but which contains both left and right flanking regions (Deletion, lane 3).

general region of both the human and mouse GR promoters, suggests that this AP-1 site may play some important, basic, functional role in regulating GR gene expression.

The putative GR AP-1 site in the human GR promoter binds to AP-1 complexes *(15)*. In this study, we demonstrated that a pan Fos antibody, directed against a highly conserved region in Fos proteins, shifted all of the complexes bound to the AP-1 site in EMSA analyses (Fig. 6A). These data indicate that the GR AP-1 site is mainly bound by the Jun/Fos complexes. Using Fos- and Jun-specific antibodies for supershift analyses, the composition of the AP-1 complexes that bound to the GR AP-1 site was determined. It was shown that FosB and Fra-2 are the major Fos members that bind to the GR AP-1 site, whereas binding of cFos is undetectable *(15)*. Very interestingly, an increase in cFos protein levels in NIH 3T3 cells efficiently stimulates

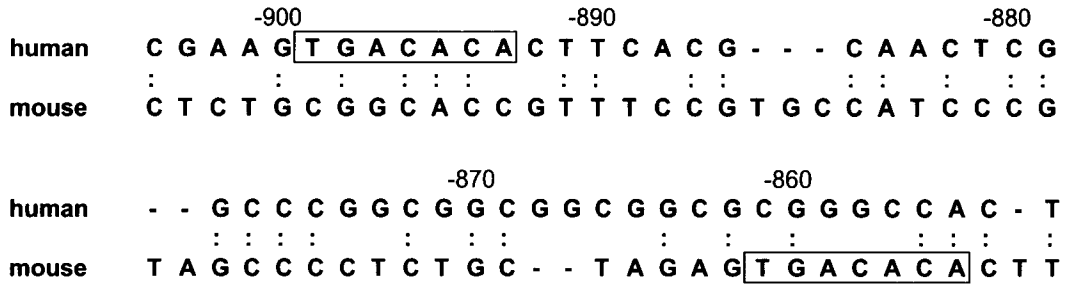


Fig. 7. Alignment of the human and mouse GR promoter sequences. The human and mouse GR promoter sequences were aligned as in Nobukuni et al. *(13)*. The human sequence is numbered as originally published in Zong et al. *(10)*. The nonconsensus AP-1 sites (TGAC<u>A</u>CA) in both promoters are boxed.

GR promoter activity via the AP-1 site (Fig. 5). These results strongly support the idea that the cFos/cJun dimer is very potent in stimulating gene transcription *(16)*. Because other Jun or Fos family members can suppress transcription from AP-1 sites in certain cell types *(16,21–23)*, it has been suggested that complex interactions of different AP-1 components in different cell types could lead to cell-type-specific regulation of the same gene. Thus, it is possible that overexpression of cFos in NIH 3T3 cells causes a redistribution of AP-1 proteins, such that FosB/Jun and/or Fra-2/Jun dimers are disrupted, resulting in cFos/Jun dimers being formed. These more active cFos/Jun dimers could then cause increased expression of the GR gene.

Using oligonucleotide-affinity chromatography, Warriar et al. *(20)* isolated two proteins that bind to the GR AP-1 site. These two proteins were subsequently identified as DNA binding autoantigens Ku80 and Ku70. However, Ku is already well known as a potential contaminant in protein purification schemes involving oligonucleotide affinity chromatography steps *(24,25)*, and it is a major factor causing nonspecific bands in electrophoretic mobility shift assays *(26)*. Therefore, these conclusions using oligonucleotide-affinity chromatography need to be viewed with caution.

Cell-cycle regulation of signal transduction pathways in higher eukaryotes has been noted since 1969. Martin et al. *(27)* observed that in cultured rat hepatoma cells, tyrosine aminotransferase (TAT) activity was induced by glucocorticoids in late G_1 and S phase, but not during G_2/M. Later, it was found that glucocorticoid binding sites increase by two- to threefold during late G_1 in synchronized HeLa cells (28). A similar increase was also observed for lymphocytes in S and post-S phase over those in G_0 and G_1 *(29)*, suggesting that the synthesis of new glucocorticoid receptors near S phase may be a general phenomenon in proliferating cells. More recent studies revealed that many cells are unresponsive to glucocorticoids during the G_2 phase of the cell cycle, further supporting the idea that some activities of the GR may be subject to cell-cycle control *(30,31)*. Our present studies clearly demonstrate that both GR protein and mRNA levels are induced when quiescent NIH 3T3 cells are stimulated by serum addition to enter G_1 from G_0 (Fig. 1); this may account for the increase in hormone binding observed during G1 and S phase *(28,29)*. However, caution must be exercised in extrapolating serum-stimulation effects to cell-cycle regulation, since cell-cycle-independent effects of serum components on signal transduction pathways can occur.

A number of studies have demonstrated that both c-*jun* *(18,32,33)* and c-*fos* *(17,34,35)* are rapidly induced by growth factors in quiescent fibroblasts. Furthermore, inhibition of Fos and Jun activities either by expression of antisense RNA *(36–38)* or by microinjection of antibodies *(39,40)* inhibits induction of cell proliferation and cell cycle progression. In view of their roles as transcriptional regulators, it seems likely that the Jun or Fos proteins are required for the expression of other cell-cycle-regulated genes, such as the GR gene. In support of this, NIH 3T3 cells, which exhibited a twofold increase in cFos protein levels also demonstrated a similar induction of the endogenous GR protein (Fig. 4) and GR promoter activity (Fig. 5). Further analysis of the GR promoter showed that the putative GR AP-1 site is required for this cFos-mediated induction of the GR gene (Fig. 5). Therefore, we propose that binding of the AP-1 protein to the AP-1 site can cause induction of the GR gene. A likely possibility is that increases in AP-1 protein levels in late G_1 and early S phase contribute to increased GR promoter activity that results in the S phase-specific increase in GR protein levels.

Materials and Methods

Cell Culture

Mouse fibroblast NIH 3T3 cells were obtained from the American Type Culture collection and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% Colorado calf serum (Colorado Serum Co., Denver, CO). The NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10) cells were maintained in the presence of additional 200 μg/mL of G418 (Geneticin, Gibco, Gaithersburg, MD). The cells were grown at 37°C in a humidified incubator under 6% CO_2.

To perform serum stimulation, the NIH 3T3 cells were cultured with medium containing 0.5% serum for 48 h. The cells were then stimulated with medium containing 20% serum for various times before they were collected.

Plasmid Constructs

The human GR promoter was a kind gift from E. Brad Thompson (UT Medical Branch, Galveston, TX) and was subcloned into the pGL3Basic vector (Promega, Madison, WI). The human GR promoter/luciferase constructs variable 5'-ends were created as described elsewhere (Breslin and Vedeckis, submitted). In vitro mutagenesis of the putative GR AP-1 site from the full-length promoter (–2738 to +19) was performed using Muta-Gene in vitro Mutagenesis Kits (Bio-Rad Laboratories, Hercules, CA). The oligonucleotides used were as follows: AP-1 mutation: 5'-GCCGAGTTGCGTGAAGCCAGTCCCTTCGAAAGGGGCTA CGG-3'; AP-1 deletion: 5'-GGGCCGAGTTGCGTGAA GCTTCGAAAGGGGCTACGGGG-3'. All mutations were confirmed by DNA sequence analysis.

For construction of the pGEM2-fos plasmid, the 1-kb *Pst*I fragment of pfos-1 (provided by Inder M. Verma, the Salk Institute, San Diego, CA) was inserted into the *Pst*I site of the pGEM2 vector (Promega). JAC. 1 was provided by Daniel Nathans (the Johns Hopkins University School of Medicine, Baltimore, MD). pL7Bgl200 was provided by Robert P. Perry (the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia). The cFos expression plasmid, CMV-fos, was provided by Tom Curran (St. Jude Children's Research Hospital, Memphis). The β-galatosidase expression plasmid pCH110 was purchased from Pharmacia Biotech Inc. (Piscataway, NJ).

Transfections

Transfections were performed by using LipofectAmine (Gibco). To generate the NIH 3T3 (cFos 3) and NIH 3T3 (cFos 10) cells, 2 μg of CMV-fos, 2 μg of pSV2-neo, and 10 μL of LipofectAmine were used/well of a 6-well plate. Twenty-four hours after transfection, G418 was added to the cells to a final concentration of 400 μg/mL. After ten d of selection, 12 single-cell clones were selected and tested for the expression of the cFos protein using Western blot analysis *(5)*. Two clones, NIH 3T3 (cFos 3) and (cFos 10), which expressed the cFos protein at an approximately twofold higher level than that of the parental cells, were chosen for further study.

GR promoter activity was studied using transient transfection; 1×10^5 cells were seeded into each well of six-well plate. The next day, 2 μg of promoter-luciferase constructs and 2 μg of the β-galactosidase expression plasmid pCH110 were transfected into either NIH 3T3, NIH 3T3 (cFos 3), or NIH 3T3 (cFos 10) cells. Forty-eight hours after transfection, promoter activities were analyzed by measuring the luciferase activity as described below.

Luciferase Assay and Western Blot Analysis

Luciferase activity was assayed with a luminometer (Dynatech, Microlite™ 2250, Dynex, Chantilly, VA) following the protocol provided by Analytical Luminescence Laboratory (Ann Arbor, MI). In transient transfections, variations in transfection efficiency were normalized using cotransfection with pCH110 and assaying β-galactosidase activity with Galacto-LightTM (TROPIX, Inc., Bedford, MA). Western blot analysis was carried out as described previously in Vig et al. *(5)*, using the monoclonal anti-GR antibody, BuGR2 *(41)*, the rabbit polyclonal anti-cFos antibody (#sc-52, Santa Cruz Biotechnology, Santa Cruz, CA), and the rabbit polyclonal anti-cJun antibody (#sc-45, Santa Cruz Biotechnology). Final results were obtained by densitometric scanning of the X-ray films with a BioMed soft laser densitometer.

RNA Purification and Ribonuclease Protection Assay

Total cellular RNA was isolated using TRI Reagent® (Molecular Research Center, INC., Cincinnati, OH). To generate riboprobes, pSP64GR (350a) and pGEM2-fos were linearized with *Sal*I, JAC. 1 was linearized with *Pvu*II, and pL7Bgl200 was linearized with *Xba*I. The linearized DNA templates were used to perform in vitro transcription using a MAXIscript kit (Ambion, Inc., Austin, TX). SP6 RNA polymerase was used to generate the GR probe, and T7 RNA polymerases were used to generate the c-*jun*, *fos*, and L7 probes. The specific activity of the L7 probe was 0.6% of that of the GR and c-*jun* probes or 1.2% of that of the *fos* probe. This allowed examination of all transcripts in the same gel lane. [^{32}P]labeled RNA probes were then hybridized with 15 μg of total RNA. Free probes were removed using 100 U/mL RNase T1 (37°C, 30 min) (Ambion, Inc.). The probes that hybridized to complementary RNA in the sample mixture were protected from ribonuclease digestion, and the reaction products were analyzed on a 6% polyacrylamide/7 *M* Urea gel as described elsewhere *(42)*. Relative mRNA levels were analyzed using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared as described elsewhere *(15)*. To study the binding activity of the putative GR AP-1 site, the deoxyoligonucleotides used in the electrophoretic mobility shift assays included: Wild Type, 5'-CCTTTCGAAG **TGACACA**CTTCACGCAACTC-3', positions -879 to -908; Mutation, 5'-CCTTTCGAAG**GGACTGG**CTTCA CGCAA CTC-3'; and Deletion, 5'-CCTTTCGAAG CTTCA CGCAACTC-3'. The electrophoretic mobility shift assays were performed essentially as previously described *(15)*. Briefly, labeled probes were incubated with 10 μg nuclear extract. For supershift assays, 2 μg of the rabbit polyclonal antipan (c)Fos antibody (#sc-253X, Santa Cruz Biotechnology, Santa Cruz, CA) were used. Samples were separated by electrophoresis on a 4% nondenatruing polyacrylamide gel. Gels were dried, and the images were analyzed with the ImageQuant program (Molecular Dynamics).

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