Transrepression of c-*jun* Gene Expression by the Glucocorticoid Receptor Requires Both AP-1 Sites in the c-*jun* Promoter

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The c-*jun* protooncogene encodes a nuclear protein, cJun, which is a major component of the AP-1 transcription factor. AP-1 regulates various aspects of cell proliferation and differentiation. As an immediate early response gene, the expression of the c-*jun* gene is affected by various extracellular stimuli, such as serum, phorbol esters, and glucocorticoids. In mouse L929 fibroblasts, dexamethasone (DEX) treatment caused a 60% reduction of c-*jun* mRNA levels. Previous studies indicated that this reduction is due to the alteration of the transcription rate of the c-*jun* gene. To further investigate the molecular mechanisms of transcriptional repression of c-*jun* by DEX, a full-length human c-*jun* promoter, from −1780 to +731, was amplified from genomic DNA using PCR and then linked to the luciferase reporter gene. To identify the regulatory elements responsible for the down-regulation, nested deletions spanning the promoter were generated, and the promoter/luciferase constructs were transiently transfected into L929 cells. Upon hormone treatment, basal activity of the full-length c-*jun* promoter was reduced by ~40%, which accounts for two-thirds of the overall down-regulation observed at the mRNA level. This reduction of c-*jun* promoter activity was abolished after deletion of the region between −1780 to −63, where two AP-1 sites (−182 and −64) are located. Site-directed deletion of these AP-1 sites reduced the basal activity of the c-*jun* promoter and prevented repression by DEX. Repression of the c-*jun* gene is due to the transrepression activity of the glucocorticoid receptor (GR), as determined using GR mutants lacking this activity. Overexpression of cJun overcame the negative effect of DEX, suggesting that down-regulation of the c-*jun* gene by hormone is mediated by the interaction between the GR and the cJun protein. These studies are the first to show that glucocorticoids can repress c-*jun* promoter activity through the AP-1 sites in the c-*jun* promoter in mouse fibroblast cells. They also suggest that inhibition of cell proliferation by glucocorticoids may be due not only to the interference with AP-1 activity on other cellular genes, but also because of a direct transcriptional suppression of c-*jun* gene expression by the GR. (Molecular Endocrinology 12: 1322–1333, 1998)

INTRODUCTION

c-*jun* is a protooncogene that encodes cJun, the major component of the activator protein-1 (AP-1) transcription factor (reviewed in Refs. 1 and 2). c-*jun* belongs to a class of cellular genes, termed immediate-early genes, whose transcription is rapidly regulated in response to a variety of external stimuli, including growth factors, cytokines, tumor promoters, UV radiation, and hormones (3–10).

Glucocorticoid treatment causes a decrease in c-*jun* mRNA levels in many cell lines, although the degrees of inhibition and kinetics are different (9–11). The effect of glucocorticoids on the c-*jun* gene is a primary effect, as it occurs rapidly and is not inhibited by cycloheximide (9). Furthermore, nuclear run-on transcription assays revealed a rapid decrease in c-*jun* gene transcription rates (9), suggesting that down-regulation of the c-*jun* gene expression occurs at the transcriptional level.

Among important regulatory elements previously identified in the c-*jun* promoter are two AP-1 sites, a proximal AP-1 site (pAP-1) located between −71 and −64 in the c-*jun* promoter (5) and a distal AP-1 site (dAP-1) located between −190 and −183 (12). Preexisting cJun homodimers and cJun/ATF-2 heterodimers can bind, respectively, to these two AP-1 sites and activate transcription (5, 12). Both AP-1 sites are involved in transcriptional regulation in response to UV irradiation (8, 12), phorbol esters (5), or the E1A product of adenovirus (7, 13). It has been well established that glucocorticoids repress genes that are

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under the positive control of the AP-1 transcription factors (reviewed in Refs. 14 and 15). The presence of these AP-1 sites within the c-*jun* promoter suggested that they may be the key elements involved in the response of the c-*jun* gene to glucocorticoids.

Based on previous studies, a transcriptional interference model has been proposed for hormone-mediated down-regulation of c-*jun* gene transcription, in which interference with AP-1 activity by the glucocorticoid receptor (GR) caused inhibition of c-*jun* gene expression (9). In this paper we show that the inhibitory effects of glucocorticoids on basal c-*jun* gene expression in mouse fibroblast cells are mediated via both the distal and proximal AP-1 sites in the promoter. Specific mutation of these AP-1 sites correlated with a significant reduction of basal promoter activity and with the loss of the glucocorticoid-mediated down-regulation. We provide evidence suggesting that repression of c-*jun* gene expression by glucocorticoids is due to the transrepression activity of the GR protein. In addition, overexpression of the cJun protein blunts the response of c-*jun* to glucocorticoids. Finally, given the role of cJun in cell proliferation (3, 4, 6, 16–18), we propose that repression of c-*jun* transcription represents an important mechanism for the antiproliferative effects of glucocorticoids.

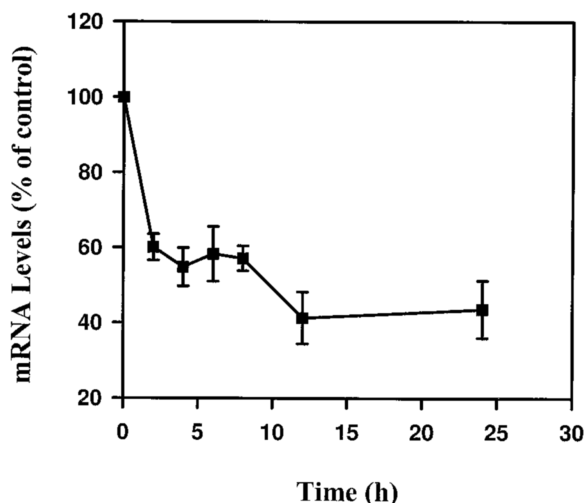

Fig. 1. DEX-Dependent Down-Regulation of c-*jun* mRNA Levels in L929 Cells

L929 cells were treated with 1 μM DEX or with ETOH vehicle only for the indicated times. Total mRNA samples were assayed using an RPA, and the PhosphorImager 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-*jun* signals. Each data point (means ± SEM) was represented as the percent of the vehicle-treated, control value for c-*jun* mRNA at each time point.

RESULTS

Down-Regulation of c-*jun* mRNA Levels by Dexamethasone (DEX)

Previously, it was shown that glucocorticoid treatment causes a dramatic down-regulation in cJun protein levels (to 10–25% of control levels) in mouse L929 fibroblasts (10). To determine whether this decrease of the cJun protein was accompanied by similar changes in the c-*jun* mRNA, total RNA was extracted from L929 cells treated for various times with ethanol (ETOH) or 1 μM DEX, a glucocorticoid agonist. The mRNA samples were assayed using a ribonuclease protection assay (RPA), and the PhosphorImager data were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The signals for mouse L7 ribosomal protein mRNA (19) obtained at each time point were used to normalize the c-*jun* signals, as L7 mRNA is not regulated by glucocorticoid treatment in L929 cells. Each data point was represented as the percent of the vehicle-treated, control value for c-*jun* mRNA at each time point.

The c-*jun* mRNA levels decreased to approximately 60% of those observed in the control cells 2 h after hormone treatment (Fig. 1). They were reduced further as the time of hormone treatment increased. This biphasic curve suggests the interesting possibility that there are two kinetic processes involved in DEX-mediated inhibition of c-*jun* gene expression. Twenty-four hours after the addition of hormone, the c-*jun* mRNA levels were only 30–40% of the control cells. Thus, DEX treatment rapidly and dramatically inhibited c-*jun* gene expression in a time-dependent manner.

Effect of DEX on the c-*jun* Promoter

Nuclear run-on transcription assays revealed that repression of the c-*jun* gene by glucocorticoids in mouse pituitary tumor cells is due to the decrease in the transcription rate (9). These results suggest that the c-*jun* promoter is involved in this glucocorticoid-mediated down-regulation. To study the role of the c-*jun* promoter in the regulation of c-*jun* gene expression, we amplified the full-length human c-*jun* promoter plus 731 bp of downstream sequences (−1780/+731) from genomic DNA. This fragment (hereafter referred to as the full-length c-*jun* promoter) was cloned upstream of a luciferase (LUC) reporter gene. The entire promoter was sequenced to ensure that the PCR amplification did not introduce any cryptic mutations within the promoter. Surprisingly, we discovered that up to 6% of the sequence of the PCR product differed from the published sequences (20, 21). We then sequenced two c-*jun* promoter fragments, which were originally isolated from human genomic libraries (generous gifts from Dr. Andrew S. Kraft, University of Colorado Health Sciences Center, Denver, CO). We confirmed that except for two nucleotides, the sequences from both the genomic DNA and the PCR products are identical. The corrected sequence has been submitted to GenBank.

To identify functional elements involved in regulating c-*jun* gene expression, we constructed a series of deletion mutations of the c-*jun* promoter with variable 5′-ends. These promoter/LUC constructs were transiently transfected into L929 cells. The activity of the full-length promoter was arbitrarily defined as 100%. Deletion from −1780 to −345 did not affect the basal transcriptional activity of the promoter (Table 1). By contrast, 70% of the basal promoter activity was lost after deletion of the region between −345 and −180. The region between −180 and −63 contributed the rest of the basal promoter activity. Therefore, the elements responsible for the basal promoter activity seem to be located between −345 and −63. We next studied DEX inhibition of the c-*jun* promoter activity. Cells transfected with the promoter/LUC constructs were treated with either ETOH or 1 μM DEX for 24 h. The promoter activity of each DNA construct in ETOH-treated cells was defined as 100%. A glucocorticoid-dependent decrease in c-*jun* promoter-driven luciferase activity was observed (Fig. 2). The reporter construct containing a full-length promoter sequence exhibited about a 40% reduction of the luciferase activity. Deletion from −1780 to −180 did not significantly change the DEX effect on the c-*jun* promoter, while further deletion to −63 abolished down-regulation of the c-*jun* promoter by DEX. Thus, it appears that the critical elements for glucocorticoid responsiveness reside in the region from −180 to −63 in the c-*jun* promoter.

Reduction of c-*jun* Promoter Activity by DEX Is Mediated by the AP-1 Sites within the Promoter

Angel *et al.* (5) identified Sp1, CTF, and AP-1 sites within the region from −130/+170. This AP-1 site (proximal AP-1 or pAP-1, 5′TGACATCA3′), located between positions −71 and −64, differs from the collagenase (consensus) AP-1 binding site by a nucleotide insertion (*underlined*). Mutational analysis revealed that this pAP-1 site plays a regulatory role in response to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induction and overexpression of the cJun protein (5). A second putative AP-1 binding site (distal AP-1 site or dAP-1 site) between positions −190 and −183 has also been detected (12). It differs from the identified AP-1 site present in the SV40 enhancer (22) by an additional base pair (5′TTACCTCA3′; *underlined*).

To study how these two AP-1 sites contribute to the basal promoter activity and the hormonal response, we specifically mutated or deleted the AP-1 sites in the context of the full-length promoter. Again, the basal promoter activity of the full-length, wild-type c-*jun* promoter was defined as 100%. Mutation of the pAP-1 site reduced the basal activity to about 60%, while deletion of this site reduced it to about 40%, of the control levels (Table 1). The dAP-1 site is also required for basal promoter activity since the c-*jun* promoter lost 40% of its activity after deletion of this site. Finally, only 22% of the promoter activity remained after both AP-1 sites were deleted. Taken together, these results demonstrate that both AP-1 sites are essential in contributing to the basal activity of the c-*jun* promoter (Table 1).

Table 1. Basal Activity of the c-*jun* Promoter

DNA Constructs (promoter/LUC)	Basal Promoter Activity (% of the full-length, wild-type promoter)
−1780/+731 (full length)	100
−952/+731	120 ± 17
−716/+731	106 ± 13
−345/+731	131 ± 15
−180/+731	28 ± 10
−63/+731	3 ± 1
−1780/+731 (wild-type)	100
−1780/+731 (pAP-1 mutation)	56 ± 8
−1780/+731 (pAP-1 deletion)	38 ± 5
−1780/+731 (dAP-1 deletion)	63 ± 11
−1780/+731 (dpAP-1 deletions)	22 ± 4

L929 cells were transfected with c-*jun* promoter deletion mutant-luciferase gene chimeric plasmids with variable 5′-ends (from −1780 to −63), or a full-length promoter containing mutation or deletion of either the pAP-1 or the dAP-1 site. Results are expressed as percent of luciferase activity driven by a full-length, wild-type promoter fragment normalized for β-galactosidase activity. Data are presented as the mean ± SEM from at least three different experiments.

The GR represses a number of different genes by interacting with transcription factors, including the AP-1 transcription factor (14, 15). Since the c-*jun* AP-1 sites play important roles in basal promoter activity, it seemed feasible that they also mediate the down-regulation of the c-*jun* gene expression by DEX. To examine whether the AP-1 sites are also the targets for repression by glucocorticoids, we transiently transfected L929 cells with promoter/LUC constructs containing either mutated or deleted AP-1 sites. Again, incubation of the transfected cells with DEX led to a significant decrease in the activity of the full-length, wild-type promoter (Fig. 3). Deletion or mutation of either the pAP-1 or the dAP-1 site alone did not significantly alter the DEX responsiveness of the promoter. By contrast, the promoter/LUC construct (dpAP-1 Dels) from which both AP-1 sites had been deleted was not repressed at all by DEX (Fig. 3). Thus, either AP-1 site within the c-*jun* promoter is sufficient to mediate DEX inhibition of c-*jun* gene expression. Only when both sites are mutated is DEX repression on c-*jun* promoter activity lost.

In addition to the upstream AP-1 sites, the c-*jun* gene contains an additional AP-1 site downstream of the transcription start site (internal AP-1, iAP-1), between positions +696 and +708 (5). This is a weak binding site that does not seem to be responsible for TPA induction or positive autoregulation (5). This AP-1 site is not involved in down-regulation of the c-*jun* promoter because deletion of it did not change the repressive effect of DEX on promoter activity (data not shown).

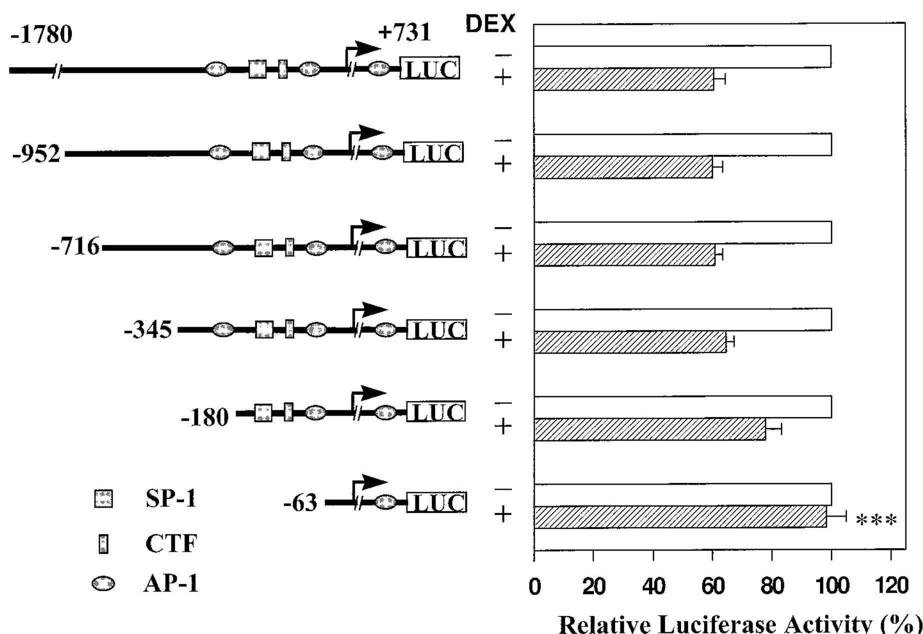

Fig. 2. Deletion Analysis of the Human c-*jun* Promoter

The *schematic diagram* represents a series of c-*jun* promoter deletion mutant-luciferase gene chimeric plasmids with variable 5′-ends (from −1780 to −63). Each construct was transiently transfected into L929 cells. Transfected cells were then treated with ETOH or DEX for 24 h before measurement of luciferase activity. Promoter activity is normalized for transfection efficiency by dividing luciferase activity by β-galactosidase activity of a cotransfected CMV-βGal reporter plasmid. The normalized luciferase activity for each construct in ETOH-treated cells is arbitrarily defined as 100%, while that in DEX-treated cells is expressed relative to the control. Results are presented as the means ± SEM of at least three independent experiments. Statistical significance was evaluated by ANOVA and was set at $P < 0.05$, when comparing the deletion mutants to the full-length c-*jun* promoter. While the Student-Newman-Keuls test using homogeneous subsets indicated that the −180 was in a subset distinct from the four longer constructs and from the −63 construct, the Dunnett two-sided *t* test comparing the full-length −1780 construct and the −180 deletion construct did not show a statistically significant difference between these two constructs. *** Indicates a significant loss of repression of the c-*jun* promoter activity ($P < 0.001$) compared with that obtained with the full-length, wild type c-*jun* promoter.

Repression of c-*jun* Gene Expression by DEX Is Strictly Dependent on the Presence of the Functional GR Protein

To study the role of the GR protein in down-regulation of c-*jun* gene expression, we analyzed the c-*jun* mRNA in the E8.2 cell, a mouse L929 fibroblast variant that does not express any endogenous GR protein (23). DEX treatment did not decrease c-*jun* mRNA levels in these cells (Fig. 4), suggesting that the functional GR protein is necessary for this process. A rat GR expression plasmid was then stably transfected into these cells to reconstitute the GR protein level. A derivative cell line, E8.2/GR3, was obtained from a single transfected cell; the expression of the rat GR protein in this line is controlled by tetracycline via the tetracycline-regulated expression system (24). Therefore, the GR protein levels can be modulated by tetracycline (Tc) (25). Forty-eight hours after removal of Tc, E8.2/GR3 cells express rat GR protein equivalent to that in wild-type L929 cells (25). In these cells, we observed a rapid down-regulation of the c-*jun* mRNA 2 h after the addition of hormone (Fig. 4). The c-*jun* mRNA levels were suppressed about 40% by DEX treatment. This pattern of down-regulation resembles that seen in the wild-type L929 cells (Fig. 1). These results indicate that functional GR protein is required for the hormone-mediated inhibition of c-*jun* gene expression.

Inhibition of c-*jun* Gene Expression by DEX Is Mediated by the Transrepression Activity of the GR

Previous studies showed that the DNA binding domain and the ligand-binding domain of the GR are essential components for the transrepression activity of this protein (26–31). Furthermore, by introducing point mutations into the DNA binding domain, Heck *et al.* (32) generated GR mutants that fully activate glucocorticoid-regulated genes but cannot repress AP-1 activity. To examine their effect on the inhibition of c-*jun* gene expression, we stably transfected GR-negative E8.2 cells with expression plasmids coding for wild-type human GR (pRShGRα and phGRSB), GR mutants (S425G, L436V) that lack transrepression activity (32), and GR$\alpha\Delta$463–473, in which 11 amino acids from the DNA binding domain were deleted. Using DNA sequence analysis, we discovered that the original S425G mutant was in fact a double mutant (S425G/

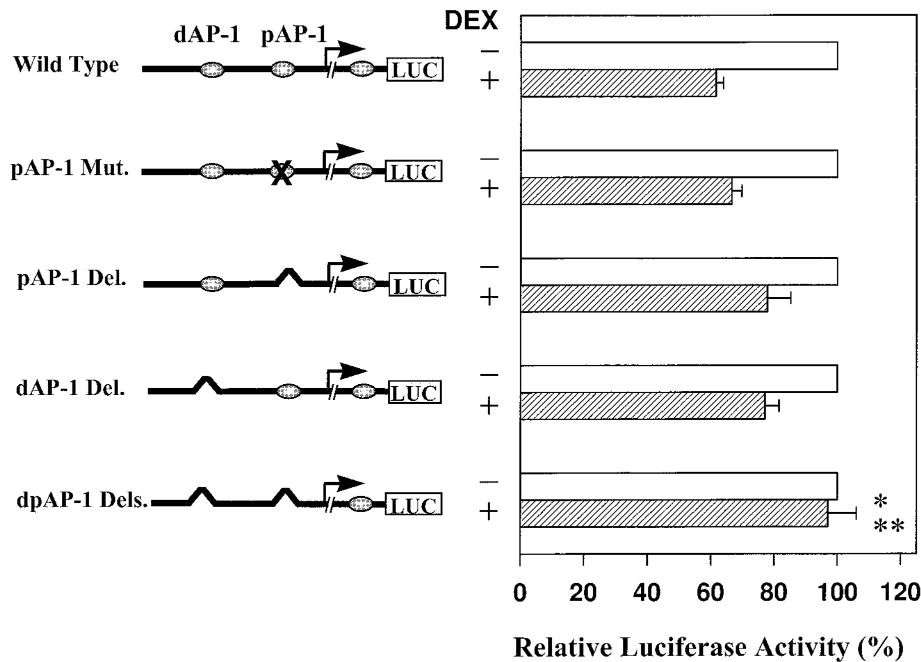

Fig. 3. Mutagenesis Analysis of the AP-1 Sites of the c-*jun* Promoter

The *schematic diagram* represents a full-length wild-type c-*jun* promoter (−1780/+731) or a full-length promoter containing mutation (Mut.) or deletion (Del.) of either the pAP-1 or the dAP-1 site. Luciferase activity was assayed and analyzed as described in the legend to Fig. 2. Results are presented as the means ± SEM. The double deletion (*asterisks*) was statistically significantly different compared with the full-length, wild-type promoter ($P < 0.01$) and the pAP-1 Mut. and pAP-1 Del. constructs ($P < 0.05$).

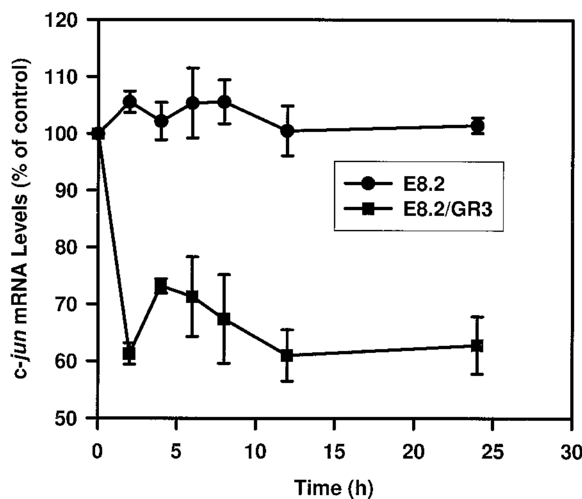

Fig. 4. Down-Regulation of c-*jun* Gene Expression Requires the Presence of the GR protein

c-*jun* mRNA levels were analyzed in DEX-treated E8.2 and E8.2/GR3 cells as described in Fig. 1. The E8.2 cells are GR-negative cells derived from mouse L929 fibroblasts. E8.2/GR3 cells express a rat GR cDNA under the control of Tc.

E427G). In our transfection experiments, expression of the GR protein was again under the control of Tc. In the presence of 1 μg/ml Tc, no GR protein was expressed in these stably transfected cells. Removal of Tc from the culture medium stimulated expression of the GR protein (Fig. 5A). In the cells expressing wild-type GR protein (GR3, pRShGRα, and phGRSB), we observed a 35–40% decline in c-*jun* mRNA levels after hormone treatment (Fig. 5B). The mutant GR containing a conservative amino acid substitution (L436V), which did not transrepress the 5× TRE TATA CAT reporter gene (32), still gave the same transrepression of the c-*jun* gene as wild-type GRs (Fig. 5B). This suggests that the repression of the endogenous c-*jun* gene promoter is opposite from that of the 5× TRE TATA CAT reporter gene, which contains a highly artificial promoter. Similar to the c-*jun* promoter, cadmium-induced expression of the heme oxygenase promoter was transrepressed by the GR (L436V) mutant (J. Alam, unpublished data), which further suggests that transrepression mutants of the GR may or may not be effective, depending upon the actual AP-1 site sequence in the DNA and the promoter context in which the AP-1 site resides. However, the c-*jun* mRNA level was not affected by DEX in the cells expressing the double-mutant GR (S425G/E427G) and the deletion mutant GRαΔ463–473. The double mutant still exhibited transactivation activity, as confirmed by transient transfection experiments with an MMTV-CAT reporter gene (J. Alam, unpublished data). On the other hand, the GRαΔ463–473 deletion mutant completely lost both transactivation and transrepression activities. Thus, these results clearly demonstrate

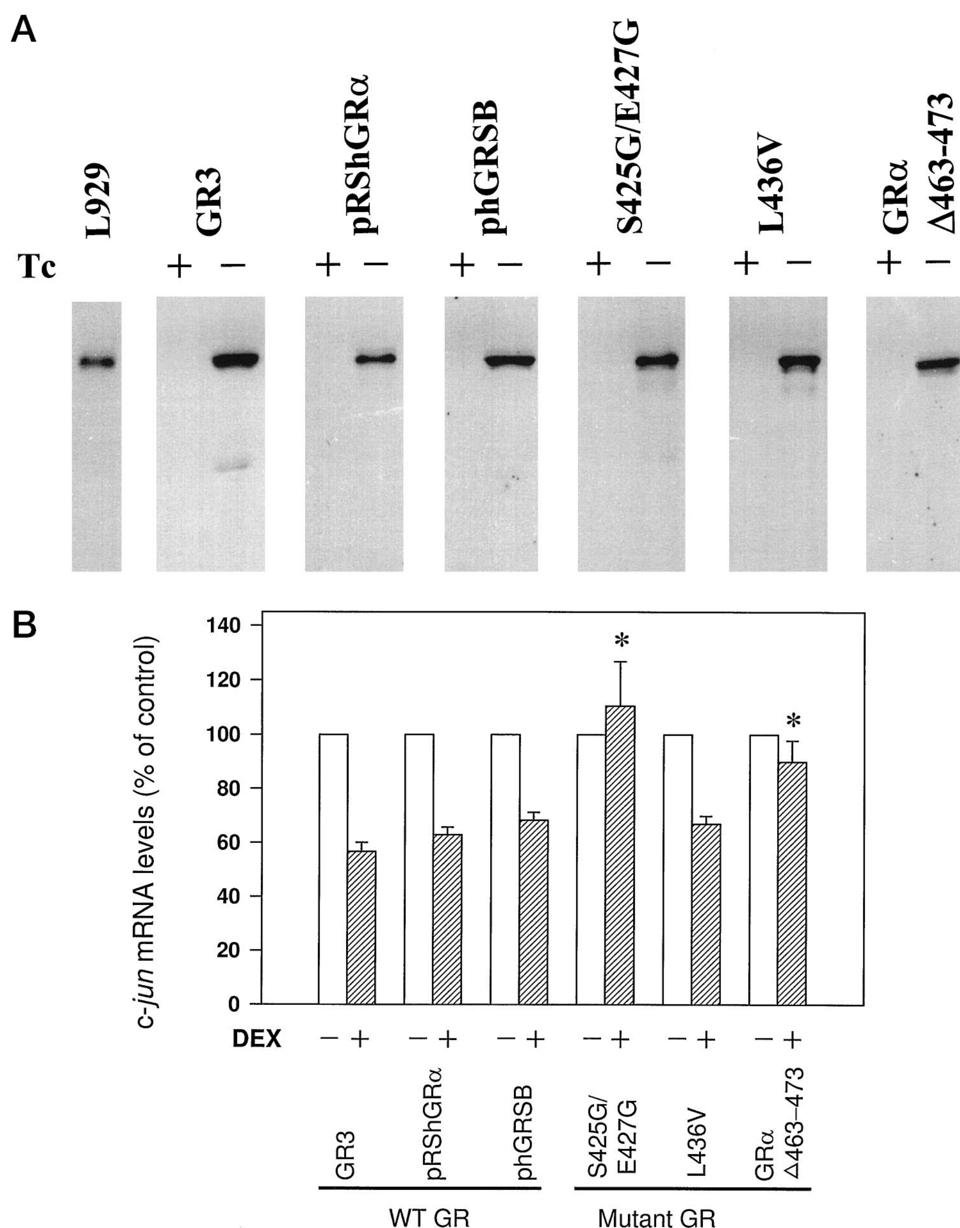

Fig. 5. c-*jun* Down-Regulation Requires the Transrepression Activity of the GR

A, Expression of the GR protein in the E8.2 cells using the Tc-regulated expression system. E8.2 cells were transfected with constructs containing cDNAs for rat wild-type (GR3), human wild-type (pRShGRα and phGRSB), transrepression-defective mutant (S425G/E427G and L436V), or GRαΔ463–473 GR proteins. Transfected cells were cultured in the presence of 1 μg/ml Tc or in the absence of Tc for various times as described in *Materials and Methods*. Whole-cell extracts were subjected to Western blot analysis using the monoclonal, BuGR2 antibody to detect the mouse and rat GR proteins and the polyclonal, PA1–512 antibody to detect the human GR protein. L929 refers to an extract obtained from wild-type L929 fibroblasts. Thirty micrograms of total cellular protein were loaded in each lane. B, The cells were cultured in the absence of Tc for various times as described in *Materials and Methods*, followed by an additional 24 h of DEX treatment. Total RNA was isolated and assayed using RPA. Twenty micrograms of total cellular RNA were used for each RPA. The c-*jun* mRNA levels in ETOH-treated cells were arbitrarily defined as 100%. The relative c-*jun* mRNA levels in DEX-treated cells are presented as the means ± SEM. *, $P < 0.05$ *vs.* wild-type pRShGRα.

that the repression of c-*jun* gene expression by glucocorticoids requires GR that is functional for its transrepression function. The prediction that this is due to interference of the AP-1 activity by the GR protein was tested next.

Overexpression of the cJun Protein Abolishes Down-Regulation of the c-*jun* Gene by DEX

Since the GR protein does not bind directly to the AP-1 site (33, 34), repression of AP-1 target genes has been

proposed to be the result of the direct interaction of the GR and cJun proteins (26, 28, 29, 35). Based on these previous studies, a similar hypothesis was suggested for down-regulation of the c-*jun* gene by hormone (9).

If the cJun protein is the molecular target in the repression of c-*jun* gene expression by DEX, then overexpression of cJun should relieve the repression. We used a cJun expression vector to examine this possibility. A full-length c-*jun* promoter/LUC plasmid was transiently transfected alone or together with a cJun expression plasmid (CMV-cJun) into mouse fibroblast NIH 3T3 cells. DEX treatment reduced the promoter activity by 35% in the control cells (Fig. 6). Furthermore, overexpression of the cJun protein alleviated the repressive effect of GR on the c-*jun* promoter. The cJun effect is dose-dependent; a slight relief of inhibition was seen with 0.5 μg of the cJun expression vector, whereas a complete prevention of inhibition was attained when a larger amount of plasmid (1.5 μg) was used (Fig. 6). Thus, production of excess cJun protein can overcome the DEX effect, suggesting that GR interferes directly with cJun activity to inhibit c-*jun* gene expression.

DISCUSSION

The regulation of transcription in eukaryotes requires the cooperative interaction between various signal transduction pathways. The GR, a member of the nuclear receptor superfamily, has been proposed to antagonize AP-1 activity. Both GR and AP-1 modulate gene transcription in response to extracellular stimuli by cross-coupling to common regulatory elements. This cross-talk involves direct protein-protein interaction, as well as DNA-binding competition for overlapping targets (28, 29, 35). In this paper we provide evidence showing a novel aspect of GR-mediated AP-1 inhibition, whereby GR represses the expression of the c-*jun* protooncogene, which encodes the major component of the AP-1 complex, cJun. To our knowledge, this is the first demonstration of GR repression of a transcription factor that is mediated by a decrease in the promoter activity of the gene coding for that transcription factor. It also conclusively shows that both AP-1 sites in the c-*jun* promoter are the targets for GR transrepression and suggests that the pathway that is disrupted is the positive autoregulation of c-*jun* gene transcription by its own gene product, the cJun protein.

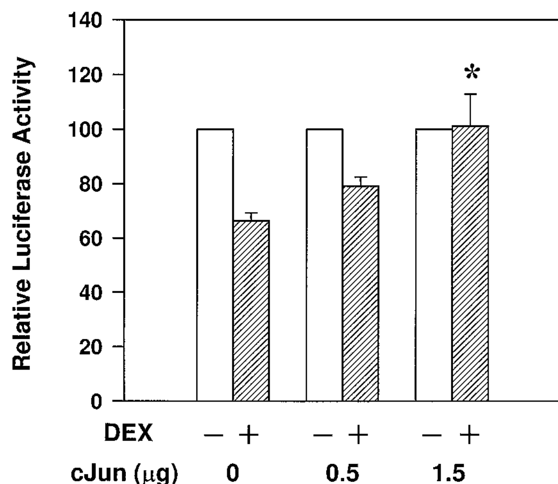

Fig. 6. Overexpression of cJun Blocks Down-Regulation of c-*jun* Promoter Activity by DEX

A cJun expression vector (CMV-cJun) was cotransfected with a full-length c-*jun*/LUC reporter gene into NIH 3T3 cells. Transfected cells were treated with DEX for 24 h before luciferase assay. Luciferase activity was assayed and analyzed as described in Fig. 2. Results are presented as the means ± SEM. *, $P < 0.05$ *vs.* control (0 μg) of transfected CMV-cJun.

The expression of c-*jun* is rapidly and dramatically down-regulated by glucocorticoids in many cell lines (9–11). Here we report that repression of c-*jun* gene expression by DEX is a direct result of decreased c-*jun* promoter activity. The 60% decrease in steady-state c-*jun* mRNA levels (Fig. 1) is at least partially responsible for the 75–90% decrease in cJun protein levels (10) seen in glucocorticoid-treated L929 cells. Deletion of proximal and distal AP-1 sites in the c-*jun* promoter abolishes the responsiveness of the c-*jun* gene to glucocorticoids, indicating that both are responsible for the down-regulation. Inhibition of the c-*jun* gene requires functional GR protein, and it is due to the transrepression activity of the GR. The inhibitory effect of glucocorticoids on the c-*jun* promoter is blocked by overexpression of the cJun protein. These data support a transcriptional interference model (Fig. 7), in which binding of the AP-1 proteins and other transcription factors to the c-*jun* promoter drives the basal transcription of the c-*jun* gene. In the presence of hormone, GR is activated by hormone binding and is released from associated proteins, such as hsp90 and hsp56 and p23. GR monomers then could form heteromeric complexes with cJun proteins, which may be prebound to the promoter. Thus, GR/cJun protein-protein interactions may modulate the AP-1 activity, finally causing down-regulation of the c-*jun* gene. It must be emphasized that glucocorticoid treatment does not universally down-regulate c-*jun* gene expression. Indeed, we (10) and others (36) have shown that the hormone increases c-*jun* mRNA and protein levels in the human CEM-C7 T-lymphoblast cell line, and cJun up-regulation is necessary for hormone-mediated apoptosis in these cells (36). Thus, it may be that in cells that respond homeostatically to the GR and cJun pathways, the hormone suppresses c-*jun* gene expression, while in those that terminally differentiate and undergo apoptosis there is a positive, reinforcing cross-talk between the two signal transduction pathways. Whether this latter situation extends past the T lymphoblast cell remains to be determined, as does

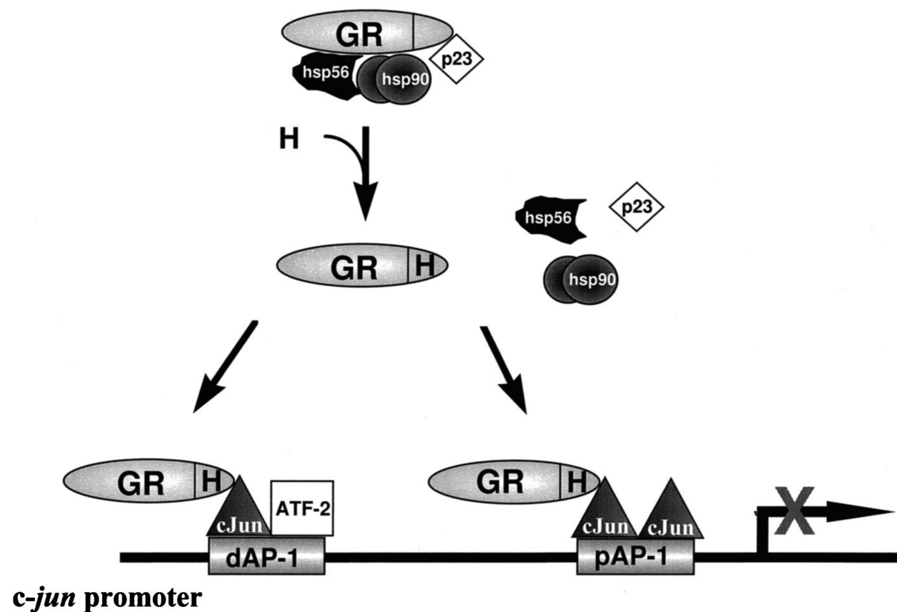

Fig. 7. Proposed Transcriptional Interference Model for Glucocorticoid-Mediated Down-Regulation of the c-*jun* Gene

The untransformed GR associates with the heat shock proteins (hsp90 and hsp56) and p23. In the hormone-free state, transcription of the c-*jun* gene occurs at a basal level. Binding of hormone (H) to GR alters the phosphorylation state of the GR and causes subunit dissociation. GR monomers may form heteromeric complexes with cJun in the nucleus. This protein-protein interaction may modify the activity of the AP-1 transcription factor, finally reducing the expression of the c-*jun* gene.

the molecular mechanism for hormone-induced up-regulation of c-*jun* gene expression.

Previous studies showed that important classes of target genes that are repressed by glucocorticoids are those that are positively regulated by AP-1 transcription factors (Reviewed in Refs. 2, 14, and 15). The best-studied examples are collagenase and stromelysin (37, 38), which are involved in the degradation of collagen and basement membrane proteins (39, 40). DEX is a potent inhibitor of collagenase gene induction, which occurs during cell proliferation or inflammation (26, 29). Analysis of the collagenase promoter indicated that the element that mediates its repression of transcription by glucocorticoids is its AP-1 site (26, 28, 29). Furthermore, these studies showed that repression occurs independent of DNA binding by the GR and involves a physical protein-protein interaction between GR and either Fos or Jun (29). Such a mechanism could account for negative effects that glucocorticoids exert on expression of the c-*jun* gene. This is supported by the following common features of repression of both collagenase and c-*jun* gene expression by hormone. First, repression is a primary effect and does not require new protein synthesis. Second, repression is mediated via the AP-1 site in the promoter. Third, repression is mediated by the GR, and a functional DNA-binding domain in the GR is required for repression of AP-1 activity. Fourth, inhibition is due to the transrepression activity of the GR protein. Finally, overexpression of the cJun protein overcomes the inhibitory effect of glucocorticoids. Thus, these results point to the fact that these two genes are regulated by glucocorticoids via similar mechanisms. However, the mere presence of an AP-1 site in a promoter does not necessarily prove that it is a target for DEX-mediated transrepression. We have shown that the GR promoter, which has an AP-1 site that binds AP-1 proteins (41) and responds positively to serum stimulation and the overexpression of cFos (42), is not the target for DEX-mediated transrepression of GR gene expression (25). Additionally, the interactions of the various members of the AP-1 family of transcription factors, and the binding of non-AP-1 transcription factors to AP-1 sites, varies tremendously depending upon the actual sequence of the AP-1 binding site in the DNA (43).

It was found that the occupancy of AP-1 sites was unchanged during induction and subsequent repression of the c-*jun* promoter by TPA and UV irradiation (44) or serum growth factors (45). Further, *in vivo* deoxyribonuclease footprinting showed that occupancy of the collagenase gene promoter AP-1 site is unaltered by DEX treatment, even though expression of the collagenase gene is strongly suppressed (46). Finally, DEX-mediated inhibition of the c-*jun* gene does not alter DNA-protein interactions at the AP-1 site *in vitro* (47). Therefore, it is likely that the GR interacts with the cJun protein while it is bound to DNA in an AP-1/DNA complex. This represents a refinement of the transcriptional interference model we presented previously for GR repression of c-*jun* gene expression, which suggested a disruption of the AP-1 complex from the AP-1 site in the promoter (9). An alternative hypothesis is that GR competes for a common coac-

tivator that is required for the activity of other transcription factors. For example, recent studies showed that P300/CBP (CREB binding protein) is required for transcriptional activation by both the GR and the AP-1 transcription factor (48). It was proposed that competition for limiting amounts of CBP might account for inhibitory actions of the GR. Whether or not there is a role for CBP in DEX-mediated transrepression of c-*jun* gene expression remains to be determined. Our studies clearly show that cJun overexpression is sufficient to overcome DEX-mediated transrepression of the c-*jun* gene. This strongly suggests that GR/cJun protein-protein interactions are important in the transrepression mechanism.

Glucocorticoids inhibit proliferation of a variety of cultured cell lines, including L929 fibroblasts (49, 50), and they are also used as antineoplastic agents (51, 52). Although antagonism between the proliferative function of AP-1 factors and the differentiative function of various nuclear receptors has been frequently noted, little is known about the mechanism by which glucocorticoids inhibit the proliferation of cells. The antiproliferative effects of glucocorticoids are believed to be mediated by the GR (50), and it could be due to inhibition of AP-1 activity (29).

Many studies of cJun function suggest that it plays an important role in cellular growth. First, the c-*jun* gene is an early-response gene that is rapidly induced in many cell types in response to mitogens such as serum, epidermal growth factor, transforming growth factor-α, and platelet-derived growth factor (3–6, 16). Second, higher c-*jun* mRNA levels were observed in logarithmically growing cells than in serum-starved cells (5). Third, the c-*jun* gene is rapidly increased during transition of fibroblasts from G_0 to G_1 (3, 16, 53, 54). Fourth, the expression of c-*jun* appears to be required for cell cycle progression in fibroblasts (4, 17, 55), and its inhibition causes a reversible cell cycle arrest (56). Finally, expression of c-*jun* in retinal tissue is high at early embryonic ages, and it decreases during development as cell proliferation declines and ceases (57). These observations support the idea that cJun may control the expression of genes involved in cellular proliferation.

The potent effects of glucocorticoids on cell proliferation may occur by regulating the expression of the AP-1-containing genes, including c-*jun*. GR interferes with the activity of the AP-1 transcription factor (26, 28, 29, 35). This interference, in turn, causes repression of the c-*jun* gene itself via the AP-1 sites within the c-*jun* promoter. Thus, in addition to proliferative genes located downstream of c-*jun*, expression of the c-*jun* gene itself may be a primary target for the antiproliferative effect of glucocorticoids. This cross-talk could represent one mechanism by which the proliferative effects of cJun are homeostatically counterbalanced by the antiproliferative effects of the glucocorticoid/GR complex, and *vice versa*.

MATERIALS AND METHODS

Cell Culture

Mouse fibroblast L929 and E8.2 cells were grown in DMEM/high glucose supplemented with 10% FBS. NIH 3T3 cells were grown in DMEM/high glucose supplemented with 10% Colorado calf serum (Colorado Serum Co., Denver, CO). The E8.2 cells transfected with either wild-type GR protein (GR3, pRShGRα, and phGRSB) or mutant GR protein (S425G/E427G, L436V, and GR$\alpha\Delta$463–473) were maintained in the presence of 1 μg/ml Tc (Sigma Chemical Co., St. Louis, MO), 200 μg/ml of G418 (Geneticin, GIBCO, Grand Island, NY), and 200 μg/ml of Hygromycin B (Sigma). All cells were grown at 37 C in a humidified incubator under 6% CO_2.

Plasmid Constructs

JAC. 1 (provided by Dr. Daniel Nathans, the Johns Hopkins University School of Medicine, Baltimore, MD) and pL7Bgl200 (provided by Dr. Robert P. Perry, the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA) were used to generate riboprobes to determine the abundance of the c-*jun* and the mouse L7 ribosomal protein mRNA transcripts, respectively. pBL3 c-*jun* −1.1/+740 and pTATACAT c-*jun* −1.6/−132 *Hin*dIII, NCO I were kindly provided by Dr. Andrew S. Kraft (University of Colorado Health Sciences Center, Denver, CO). The tTA (tet repressor) expression plasmid, pUHD15.1 neo, and the target vector, pUHD10–3, were provided by Dr. H. Bujard (University of Heidelberg, Heidelberg, Germany). Wild-type human GR (pRShGRα and phGRSB) and the mutants (L436V and S425G), provided by Dr. Andrew C. B. Cato (Forschungszentrum Karlsruhe GmbH, Karlsruhe, Germany), were digested with *Kpn*I and then filled in with T4 DNA polymerase. The DNAs were further digested with *Dra*I. The 2.5-kb fragment was cloned into pUHDBG (25), which had been digested with *Bam*HI, treated with calf intestinal alkaline phosphatase, and then filled in with the Klenow fragment of DNA polymerase. Construction of the GR$\alpha\Delta$463–473 plasmid will be described elsewhere (J. Alam, in preparation). The cJun expression plasmid, CMV-jun, was provided by Dr. Tom Curran (St. Jude Children's Research Hospital, Memphis, TN). CMV-βGal, a β-galactosidase expression plasmid, was provided by Dr. Grant R. MacGregor (Baylor College of Medicine, Houston, TX). The luciferase reporter gene, pGL3-Basic vector, was purchased from Promega (Madison, WI), and pBluescript II SK^- ($pBSSK^-$) was purchased from Stratagene (La Jolla, CA). The puromycin-*N*-acetyl transferase expression plasmid, pPUR, was purchased from CLONTECH (Palo Alto, CA).

PCR

For PCR, 100 ng human genomic DNA were used. The sense oligonucleotide was: 5′-GAGAATTCCAAGTTCAGAAGCAG-3′; the antisense oligonucleotide was: 5′-GAGCTACCCG-GCTTTGAAAAGT-3′. An *Xho*I half-site was added to the 5′-end of each oligonucleotide. The genomic DNA was denatured at 94 C for 2 min. Amplification was performed at 94 C for 10 sec, at 65 C for 30 sec, at 68 C for 2 min for 10 cycles; and then at 94 C for 10 sec, at 65 C for 30 sec, at 68 C for 2 min plus cycle elongation of 20 sec for each cycle (*e. g*. cycle no. 11 has in addition 20 sec; cycle no. 12 has in addition 40 sec, etc.) for 20 cycles; and, finally, at 68 C for 7 min. The polymerase from the Expand Long Template PCR System (Boehringer Mannheim, Indianapolis, IN) and GeneAmp 10× PCR Buffer II and $MgCl_2$ Solution (Perkin Elmer, Foster City, CA) were used to perform the reaction. The resulting fragment (−1780 to +731) was ligated, digested with *Xho*I, and then cloned into the *Xho*I site of the pGL3 basic vector (Promega, Madison, WI). The entire promoter

was sequenced using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Arlington Heights, IL).

Construction of Progressive Promoter-Luciferase Plasmid Deletions

The −1780/+730 promoter/LUC plasmid was digested with *Mlu* I. The 5′-overhang was filled in with deoxy thioderivatives by Klenow polymerase. The DNA was then digested with *Avr* II. The double digested DNA was treated with Exonuclease III (Stratagene, La Jolla, CA) for 1–8 min. Mung Bean nuclease (Stratagene) was used to create blunt ends. The DNA was ligated and the deletion promoter/LUC constructs were confirmed by DNA sequence analysis.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the Muta-Gene *in vitro* Mutagenesis Kit (Bio-Rad Laboratories, Hercules, CA). The oligonucleotides used were as follows: pAP-1 mutation: 5′-ATAGCCCATGGTGGATCCCCAAGGCCT-3′; pAP-1 deletion: 5′-CCTAAAAATAGCCCACCCCAAGGCCT-TCCC-3′; dAP-1 deletion: 5′-GGAGGCTCACGGGTCGTC-CGCTGCCCTC-3′. All mutations were confirmed by DNA sequence analysis.

Transfection

Transient transfections were performed by using LipofectAmine (GIBCO, Grand Island, NY) in six-well plates. L929 cells were transiently transfected with 3 μg c-*jun* promoter construct and 1 μg CMV-βGal plasmid per well of the six-well plate. Twenty hours after transfection, DEX was added to the cells to a final concentration of 1 μM for an additional 24 h. Promoter activities were determined by measuring the luciferase activity, which was assayed with a luminometer (Microlite 2250, Dynex Technologies, Chantilly, VA) following the protocol provided by Analytical Luminescence Laboratory (San Diego, CA). Variations in transfection efficiency were normalized by assaying β-galactosidase activity with Galacto-Light (TROPIX, Inc., Bedford, MA) (58). In NIH 3T3 cells, a total of 4 μg DNA was used, which consisted of 2 μg promoter-luciferase plasmid, 0.5 μg CMV-βGal, and 0.5 or 1.5 μg CMV-cJun expression plasmid. pBluescript II SK$^-$ (pBSSK$^-$, Stratagene) was used as carrier DNA to keep the amount of total DNA constant. The transfected cells were treated with DEX and the luciferase activity was assayed to determine the promoter activity.

Using the $CaPO_4$ precipitation technique (59), 13 μg of the pUHDBG/pRShGRα, pUHDBG/phGRSB, pUHDBG/S425G, or pUHDBG/L436V plasmids were introduced into a 10-cm dish of E8.2 T4 cells, which express an appropriate amount of tTA protein (25). Two micrograms of the puromycin-*N*-acetyl transferase expression plasmid, pPUR, was also cotransfected into the cells. Puromycin-resistant clones were screened for the expression of the GR protein using Western blot analysis.

Western Blot Analysis

Cells were cultured in the absence of Tc for 24 h (GR3), 48 h (pRShGRα, phGRSB, L436V, and GR$\alpha\Delta$463–473), or 96 h (S425G/E427G). They were then treated with either ETOH only or 1 μM DEX for an additional 24 h. Whole-cell extracts were prepared from the same flask of cells treated with ETOH. Additionally, whole-cell extracts were also prepared from cells cultured in the presence of Tc. The protein samples were subjected to Western blot analysis as described previously (9). The PA1–512 antibody (Affinity BioReagents, Golden, CO) and the BuGR2 antibody (60) were used to detect the human and rat GR proteins, respectively.

RNA Purification and Ribonuclease Protection Assay

Total cellular RNA was isolated using TRI Reagent (Molecular Research Center, Inc.). To generate riboprobes, JAC.1 was linearized with *Pvu*II, and pL7BgI200 was linearized with *Xba*I. The linearized DNA templates were used to perform *in vitro* transcription using a MAXIscript kit (Ambion, Inc., Austin, TX). T7 RNA polymerases were used to generate both the c-*jun* and L7 probes. The specific activity of the L7 probe was 0.6% of that of the c-*jun* probe because of the difference in the expression levels of these two RNAs, which were quantified in the same gel lanes. ^{32}P-labeled RNA probes were then hybridized with 15–20 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 (61).

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