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# Glucocorticoid Regulation of Corticotropin-Releasing Factor$_1$ Receptor Expression in Pituitary-Derived AtT-20 Cells

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**SUMMARY**

Corticotropin-releasing factor (CRF) receptors represent one of the primary sites for negative feedback of the pituitary by adrenocortical glucocorticoid hormones; however, the molecular mechanisms involved have yet to be elucidated. The present study examines the mechanisms by which glucocorticoids regulate CRF-R1 expression in the pituitary cell line, AtT-20. Treatment of these cells with dexamethasone resulted in a concentration- and time-dependent inhibition of CRF-R1 mRNA that was significant by 1 hr and maximal after 4 hr. Levels of CRF-R1 mRNA then returned to control levels after 24 hr. Similar changes were observed when the cells were treated with corticosterone. Pro-opiomelanocortin mRNA was also decreased after dexamethasone pretreatment; however, the time course was much slower with a significant effect only detected after 6 hr. Further analysis of the mechanisms that mediate glucocorticoid regulation of CRF-R1 mRNA was conducted. These studies demonstrated that glucocorticoid incubation significantly decreases the rate of CRF-R1 gene transcription, as determined by nuclear run-on analysis. In addition, the results demonstrate that glucocorticoid incubation significantly decreases CRF-R1 mRNA stability by approximately 50%. The down-regulation of CRF-R1 mRNA was dependent on *de novo* protein synthesis, as it was blocked by pretreatment with cycloheximide. This represents a novel mechanism for glucocorticoid negative feedback regulation of CRF-R1 expression.

Glucocorticoids are known to have a wide array of physiological actions, including cardiovascular, respiratory, immunological, reproductive, and neurobehavioral effects (1, 2). The release of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal axis, and glucocorticoids, in turn, exert a strong, negative feedback effect on this neuroendocrine loop (3–5). Dysfunction of the hypothalamic-pituitary-adrenal axis is associated with and contributes to several psychiatric and endocrine disorders (6, 7), and a great deal of interest has focused on identification of the mechanisms, at the molecular level, that mediate feedback regulation of this neuroendocrine axis.

A primary site of action for glucocorticoid negative feedback, in addition to the hypothalamus and hippocampus in brain, are the corticotrophic cells in anterior pituitary (5, 6). Stimulation of these cells by CRF results in the release of ACTH, which provides the principal stimulus for the synthesis and release of glucocorticoids from the adrenal cortex (8). Glucocorticoid negative feedback of corticotrophic cell function occurs at several levels, but the best characterized are the synthesis and release of ACTH (3, 4, 9–11) and expression of CRF receptors (5, 12, 13). Synthesis of ACTH and its precursor, POMC, is negatively regulated by glucocorticoids (9, 14–16). This occurs by decreasing the rate of POMC gene transcription (15, 17, 18). The level of CRF receptors is also decreased by glucocorticoids, but the mechanisms that mediate the down-regulation of these receptors have not been identified (5, 12, 13, 19).

Identification of the CRF receptor expressed in the pituitary makes it feasible to study the mechanisms that mediate glucocorticoid regulation of this receptor at the molecular level. The CRF receptor expressed in corticotrophic cells, referred to as CRF-R1, is positively coupled via a stimulatory G protein to the cAMP second messenger system (20). Recent reports have demonstrated that glucocorticoid treatment decreases levels of CRF-R1 mRNA in pituitary primary cultures (13, 21). In the present study, we used a pituitary cell line, AtT-20, to study the mechanisms that underlie glucocorticoid regulation of CRF-R1 expression. This transformed cell line exhibits many of the properties of corticotrophs, which makes it a good model system in which to extend the previous studies. The results demonstrate a novel, complex effect of glucocorticoids on CRF-R1 mRNA stability and gene transcription rate.

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**ABBREVIATIONS:** CRF, corticotropin-releasing factor; ACTH, adrenocorticotropic hormone; POMC, pro-opiomelanocortin; SDS, sodium dodecyl sulfate; SSPE, standard saline/phosphate/EDTA; bp, base pair(s).

## Materials and Methods

**Cell culture.** AtT-20 cells were grown in Petri dishes (100 × 20 mm) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin/streptomycin (100 μ/ml). The cells were split at a ratio of 1:5 and grown for 4–5 days. Fresh media containing dexamethasone (0.2 μM; Sigma Chemical, St. Louis, MO) was then added for the times indicated. To estimate the effects of dexamethasone on cell doubling, viable cell counts were performed by adding 0.2% trypan blue stain and counting the cells using a hemacytometer. For mRNA stability analysis, the cells were further incubated with the transcription inhibitor, actinomycin D (2 μg/ml; GIBCO, Grand Island, NY) for up to 4 hr. The influence of protein synthesis inhibition was determined by addition of cycloheximide (100 μg/ml) added 2 hr before addition of the stimulators. We have previously demonstrated in cell culture that, under these conditions, cycloheximide addition results in >95% inhibition of protein synthesis (data not shown). The reaction was stopped by the addition of ice-cold 4 M guanidine thiocyanate, 25 mM sodium acetate buffer containing 0.5% 2-mercaptoethanol, and the cells were harvested.

**RNase Protection Analysis.** RNA was prepared by scraping cells in 4 M guanidine isothiocyanate, 25 mM sodium acetate buffer containing 0.5% 2-mercaptoethanol, followed by centrifugation at 150,000 × *g* at 20° for 21 hr through a 5.7-M cesium chloride gradient. The RNA pellet was resuspended in 0.3 M sodium acetate, pH 5.2, and precipitated in ethanol. The concentration was determined by using spectrophotometry. The riboprobes were prepared by polymerase chain reaction amplification of a 425-bp fragment of the mouse CRF-R1 (corresponding to bp 614–bp 1039) from mouse CATH.a cell cDNA. This fragment was then cloned into pCR II (InVitrogen, San Diego, CA). The vector was linearized, and SP6 RNA polymerase (New England Biolabs, Beverly, MA) was used to synthesize $^{32}$P-labeled antisense riboprobes as previously described (22, 23). This technique was also used to prepare a POMC riboprobe (246-bp fragment of the mouse POMC gene corresponding to bp 661–bp 907). For RNase protection analysis (22, 23), 25 μg of total cellular RNA was hybridized with [$^{32}$P]-labeled CRF-R1 riboprobe ($10^5$ cpm/sample) overnight at 63°. The samples were then incubated with RNase A and T1 (Boehringer-Mannheim Biochemicals, Indianapolis, IN) for 45 min at 37° followed by the addition of proteinase K (Boehringer) and SDS for a further 15 min. The protected double-stranded RNA fragments were then precipitated and run on a 6% polyacrylamide gel containing urea. Receptor mRNA half-life studies were performed as previously described (22, 23). The cells were incubated in the presence or absence of dexamethasone (0.2 μM) for the times indicated, followed by the addition of the transcription inhibitor, actinomycin D (2 μg/ml). Cells were harvested at different time periods (1–2.5 hr), the RNA was isolated, and CRF-R1 mRNA was determined by using an RNase protection assay.

**Nuclear run-on analysis.** Nuclei from cells treated with vehicle or dexamethasone (0.2 μM) were isolated according to the method of Kiely *et al.* (24), and nuclear run-on analysis was performed as previously described (22, 23). Isolated nuclei were then incubated for 30 min at 30° in a transcription mixture containing 1 mM unlabeled ATP, CTP, and GTP, 0.7 mM dithiothreitol, and 250 μCi of [$^{32}$P]-UTP. After the addition of DNase 1 (20 units; Stratagene, La Jolla, CA) and 4.6 μl of 100 mM $CaCl_2$, the nuclei were incubated for a further 10 min, and newly transcribed RNA was extracted using the method of Greenberg and Bender (25). The radiolabeled RNA was denatured and hybridized to CRF-R1, vector, or cyclophilin cDNA immobilized on nylon membranes (approximately 5 μg/slot; ICN Biomedicals, Cleveland, OH) at 42° for 3 days in hybridization buffer [50% formamide, 5 × SSPE (20 × stock; 3 M NaCl, 200 mM $NaH_2PO_4$, 20 mM $Na_2EDTA$, pH 7.4)], 5 × Denhardt's solution (100 × stock; 2% Ficoll 400, 2% polyvinylpyrrollidine, 2% bovine serum albumin), 4 mg salmon sperm and 1% SDS. Filters were washed in 0.2 × SSPE followed by 0.1 × SSPE (both containing 0.1% SDS), dried, and subjected to autoradiography.

**Data analysis.** All data are reported as the mean ± standard error. In most cases, significance testing was carried out by using Student's *t* test. Significance was considered as $p < 0.05$.

## Results

**Regulation of CRF-R1 mRNA by dexamethasone in AtT-20 cells.** Levels of CRF-R1 mRNA were measured in the AtT-20 cells by using RNase protection analysis as previously described (26). Incubation of AtT-20 cells with the potent synthetic glucocorticoid, dexamethasone, resulted in a concentration-dependent down-regulation of CRF-R1 mRNA (Fig. 1) with an $IC_{50}$ value of 3.6 ± 1.4 nM (mean ± standard error, $n = 3$). The time-dependent decrease was significant within 1 hr and maximal after 4 hr (Fig. 2). Furthermore, CRF-R1 mRNA returned to basal levels after 24 hr of continued treatment with dexamethasone. A viable cell count revealed that glucocorticoid treatment had no significant effect on cell doubling time (94 ± 9.8% of control, $n = 9$). A similar time-response curve was observed when the cells were treated with corticosterone, the glucocorticoid active in mice (Fig. 2). As a control, actin levels were also measured after dexamethasone (0.2 μM) treatment. No significant changes in actin mRNA levels were observed at any time (Table 1). Similarly, cyclophilin mRNA was unaffected after a 4-hr treatment with dexamethasone (88 ± 5.4% of control, $n = 4$).

For comparison, we also examined glucocorticoid regulation of POMC, the precursor of ACTH. Incubation with dexamethasone decreased POMC mRNA levels (Fig. 3), as previously reported (14, 16); however, the time course of the response was much slower than that for CRF-R1 mRNA. Significant changes were not observed until after 6 hr of glucocorticoid treatment. In addition, levels of POMC mRNA remained decreased after 24 hr of treatment, which demon-

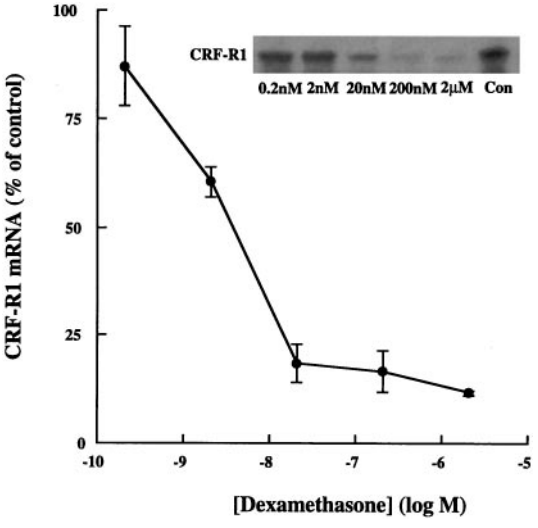


**Fig. 1.** Regulation of CRF-R1 mRNA by dexamethasone in AtT-20 cells. AtT-20 cells were incubated with increasing concentrations of dexamethasone for 4 hr. RNA was then extracted, and levels of CRF-R1 mRNA were determined by using RNase protection analysis. Representative autoradiograms from each treatment condition are shown. Levels of CRF-R1 mRNA were quantified by using densitometry. The results are expressed as the percentage of control and are the mean ± standard error of three separate experiments. *, $p < 0.05$ compared with control.

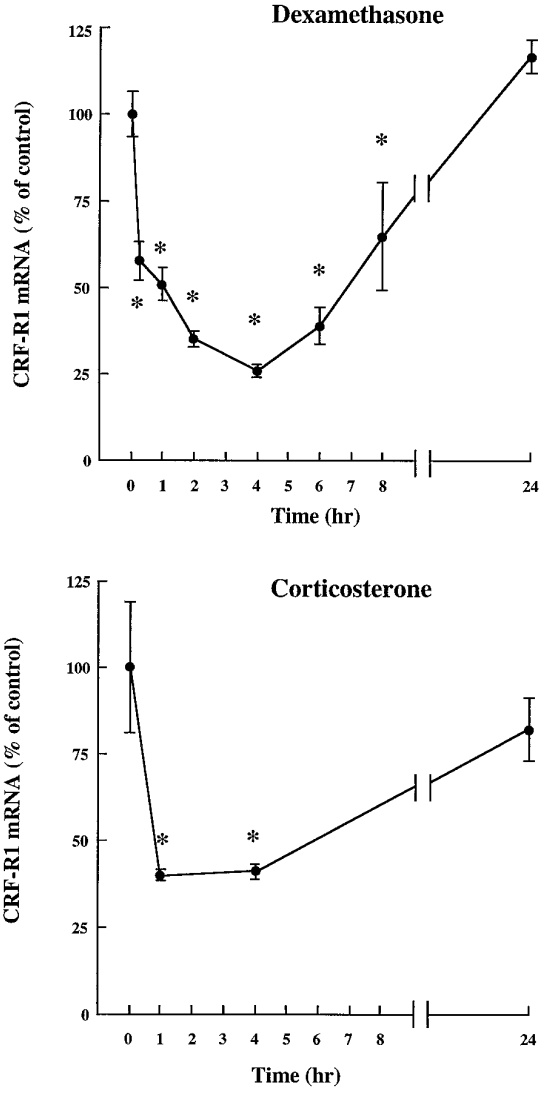


**Fig. 2.** Time course of dexamethasone regulation of CRF-R1 mRNA. AtT-20 cells were incubated with dexamethasone (0.2 $\mu$M) or corticosterone (0.4 $\mu$M) for the times indicated. RNA was then extracted, and levels of CRF-R1 mRNA were determined by using RNase protection analysis. Levels of CRF-R1 mRNA were quantified by using densitometry. The results are expressed as the percentage of control and are the mean ± standard error of three to five separate experiments. *, $p < 0.05$ compared with control.

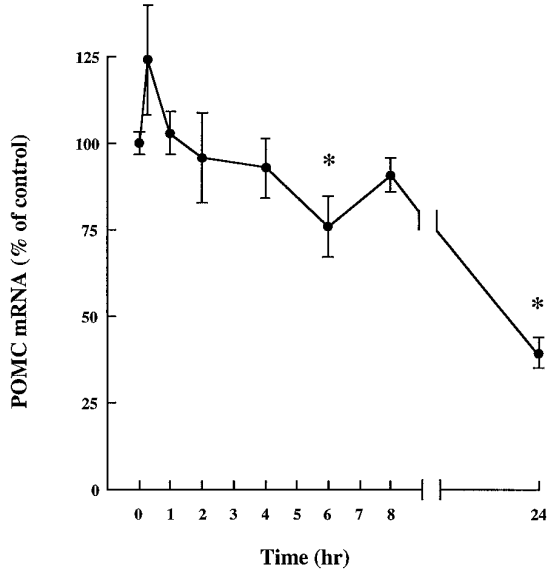


**Fig. 3.** Regulation of POMC mRNA by dexamethasone in AtT-20 cells. AtT-20 cells were incubated with dexamethasone (0.2 $\mu$M) for the times indicated. RNA was then extracted, and levels of POMC mRNA were determined by using RNase protection analysis. Representative autoradiograms from each treatment condition are shown. Levels of CRF-R1 mRNA were quantified by using densitometry. The results are expressed as the percentage of control and are the mean ± standard error of three separate experiments. *, $p < 0.05$ compared with control.

strates that reversal of the glucocorticoid regulation of CRF-R1 is specific to the receptor mRNA.

**Analysis of CRF-R1 gene transcription rate and mRNA stability.** To determine the mechanism(s) underlying the down-regulation of CRF-R1 mRNA, the effect of dexamethasone on CRF-R1 gene transcription rate and mRNA stability were determined. CRF-R1 gene transcription was determined by nuclear run-on analysis. Incubation of AtT-20 cells with dexamethasone for 2 hr significantly decreased the CRF-R1 transcription rate by approximately 30% (Fig. 4). These results demonstrate that changes in gene transcription contribute to the dexamethasone-induced down-regulation of CRF-R1 mRNA in AtT-20 cells.

The stability of CRF-R1 mRNA was determined by measuring the rate of mRNA decay after the addition of the transcription inhibitor, actinomycin D. The half-life of CRF-R1 mRNA in the AtT-20 cells was determined to be 158 ± 1.0 min (mean ± standard error, $n = 3$) (Fig. 5). Treatment with dexamethasone for 2 hr significantly decreased the half-life of CRF-R1 mRNA by approximately 50% (73 ± 8.2 min, mean ± standard error, $n = 3$). This indicates that the glucocorticoid-induced down-regulation of CRF-R1 mRNA was also, at least in part, caused by a decrease in mRNA stability.

**Requirement for *de novo* protein synthesis.** To determine whether glucocorticoid-induced down-regulation of CRF-R1 mRNA is dependent on *de novo* protein synthesis, AtT-20 cells were pretreated with cycloheximide, an inhibitor of protein synthesis, for 2 hr before the addition of dexamethasone. Incubation of AtT-20 cells with cycloheximide alone had no significant effect on CRF-R1 mRNA. However, cycloheximide pretreatment completely blocked the dexamethasone-induced down-regulation of CRF-R1 mRNA, which suggests that this effect is dependent on the synthesis of additional proteins (Fig. 6).

TABLE 1

**Effect of glucocorticoid treatment on actin mRNA levels**

Dexamethasone (0.2 $\mu$M) was added for the times indicated, RNA was extracted, and levels of actin mRNA were determined by using RNase protection analysis. The results are expressed as the percentage of control and are the mean ± standard error.

| Time (hr) | 1 | 2 | 6 | 8 | 24 |
|---|---|---|---|---|---|
| % of control | 83.2 ± 3.6 | 114.6 | 105.1 ± 12.7 | 112.6 ± 4.4 | 82.5 ± 10.0 |
| *n* | 2 | 1 | 2 | 2 | 2 |

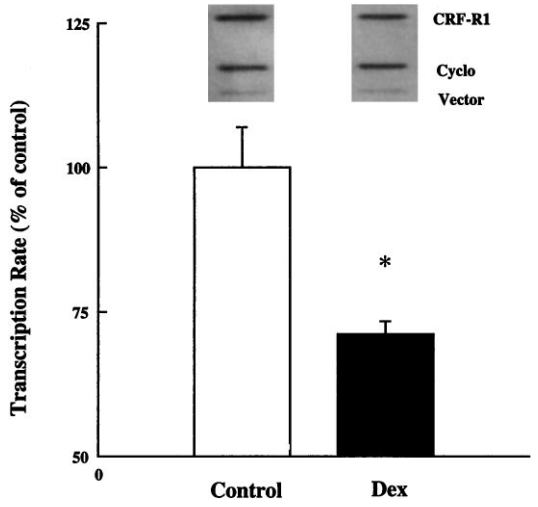


**Fig. 4.** Determination of CRF-R1 gene transcription rate. AtT-20 cells were incubated in the absence or presence of dexamethasone (0.2 $\mu$M) for 2 hr. Cell nuclei were isolated, and transcription elongation was allowed to continue in the presence of [$^{32}$P]-UTP with unlabeled ATP, CTP, and GTP. The radiolabeled nascent RNA was then isolated and hybridized to CRF, cyclophilin, and vector cDNA (5 $\mu$g), which was immobilized on nylon filters. The labeled filters were then washed and subjected to autoradiography. Representative autoradiograms for each condition are shown. The level of radioactivity in each band was quantified by using densitometry. The results were normalized to cyclophilin and are presented as the percentage of control (mean ± standard error of three separate determinations).

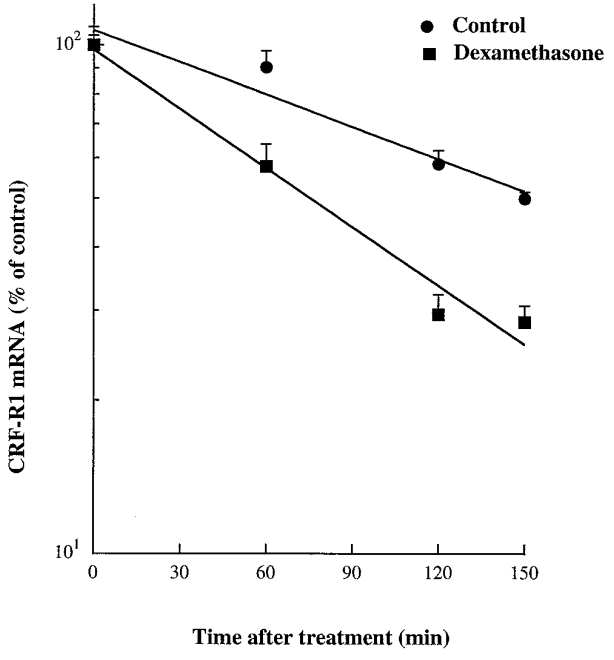


**Fig. 5.** Determination of CRF-R1 mRNA stability. AtT-20 cells were incubated in the absence (●) or presence of 0.2 $\mu$M dexamethasone (■) for 2 hr, followed by the addition of actinomycin D. The cells were then harvested at different time intervals over a 2.5-hr period. RNA was extracted, and levels of CRF-R1 mRNA were determined by using RNase protection analysis. CRF-R1 mRNA levels were quantified by using densitometry. The results are expressed as the percentage of control (mean ± standard error, $n = 3$) and are plotted on a log scale versus time.

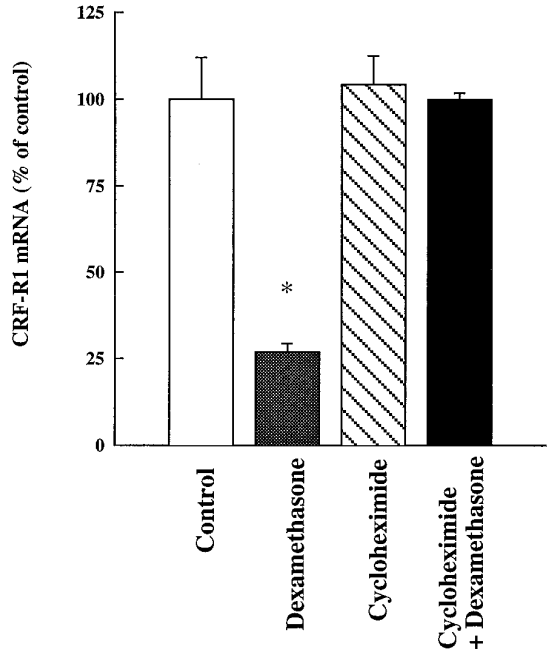


**Fig. 6.** Influence of cycloheximide on dexamethasone regulation of CRF-R1 mRNA. Cells were pretreated with cycloheximide for 2 hr, followed by dexamethasone (0.2 $\mu$M) or vehicle for 4 hr. RNA was then extracted, and CRF-R1 mRNA levels were determined by using RNase protection analysis. The results are presented as the percentage of control. *Bar,* mean ± standard error of four separate experiments; *, $p < 0.05$ compared with control.

## Discussion

The focus of the present study was to examine the mechanisms that underlie glucocorticoid feedback inhibition of CRF-R1 expression in pituitary-derived AtT-20 cells. The results demonstrate that incubation of cells with glucocorticoids results in a dose- and time-dependent down-regulation of CRF-R1 mRNA. The decrease is rapid and is similar to the time course for glucocorticoid-induced down-regulation of CRF-R1 mRNA in primary cultures of rat pituitary (13, 21). The effect was also transient, as reported in one of the studies conducted in primary cultures (21). The time course for glucocorticoid inhibition of POMC mRNA in AtT-20 cells is slower and more long-lasting (i.e., significant after 6 hr and remaining decreased after 24 hr), which is in agreement with previous reports (14, 16). These similarities suggest that the AtT-20 cells are a relatively good model for studying the mechanisms that mediate glucocorticoid regulation of CRF-R1 mRNA.

The best-characterized mechanism by which glucocorticoids influence the expression of proteins is via effects on gene transcription. Glucocorticoids bind to and activate cytoplasmic receptors, which results in the translocation of the receptor to the nucleus, in which it acts as a DNA-binding transcription factor. Steroid receptors have been shown to both stimulate (27, 28) and inhibit gene expression (15, 17, 24, 27, 29) via regulation of the gene transcription rate (15, 17, 24). The results of the present study demonstrate that glucocorticoid inhibition of CRF-R1 expression is mediated, in part, by down-regulation of the CRF-R1 gene transcription rate. Glucocorticoid-induced inhibition of POMC mRNA levels is also reported to be caused by down-regulation of gene transcription (15–17). This effect is reported to occur via

binding of the glucocorticoid receptor to a specific DNA sequence, the negative glucocorticoid response element (18). It is possible that the CRF-R1 promotor also contains a negative glucocorticoid response element, although the sequence of the gene has not yet been characterized.

An alternative mechanism for glucocorticoid inhibition of CRF-R1 gene transcription, based on the complete inhibition of the decrease observed after cycloheximide treatment, is that this effect is dependent on induction of a transcriptional repressor. There are several possible inducible repressors that may influence CRF-R1, including members of the cAMP response element modulator and/or Fos/Jun family of transcription factors. Indeed, recent evidence has demonstrated the potential for cross-talk between the glucocorticoid receptor and the Fos/Jun signaling pathway (30–32). These studies report that expression of the glucocorticoid receptor results in the blockade of *AP-1*-mediated gene activation and that expression of Jun blunts glucocorticoid receptor-mediated gene expression. It is possible that the basal transcription rate of CRF-R1 is dependent on a certain level of Fos/Jun activity and that activation of glucocorticoid receptors inhibits this activity. Characterization of the CRF-R1 promotor region and studies of glucocorticoid regulation of these potential transcription repressors are needed to further define the molecular mechanisms that mediate glucocorticoid inhibition of CRF-R1 expression.

In addition to regulation of CRF-R1 gene transcription, we also observed that glucocorticoid incubation decreased the half-life of CRF-R1 mRNA. Changes in mRNA stability in response to incubation with receptor agonist treatments have been reported for several other receptor systems (33–35). Although there are no previous reports that glucocorticoids destabilize G protein-coupled receptor mRNA, another study demonstrates that pulmonary surfactant protein A mRNA stability is decreased by glucocorticoid treatment (36). The stability of mRNA is influenced by several mechanisms, including the regulation of proteins that bind to the 3′ untranslated region and thereby destabilize the transcripts. Port *et al.* (37) have reported that agonist-induced down-regulation of β-adrenergic mRNA is accompanied by induction of a protein that binds to and, subsequently, might destabilize receptor mRNA. The same protein has been shown to bind to transcripts of other G protein-linked receptors that undergo agonist-induced destabilization (38). In the present study, glucocorticoid-mediated down-regulation of CRF-R1 mRNA in AtT-20 cells was shown to be dependent on *de novo* protein synthesis, which suggests that this effect may also be mediated by the induction of a protein that influences mRNA stability.

Evidence from the study by Port *et al.* (37) suggests that potential binding sites for the mRNA destabilizing proteins within the 3′-untranslated region are AU-rich and, in particular, have the consensus sequence AUUUA. This consensus sequence is also found four times within the 3′-untranslated region of the surfactant protein A mRNA and could contribute to the glucocorticoid regulation of its stability (36).The CRF-R1 3′-untranslated region also contains one of these consensus sites, which could be involved in the observed glucocortcoid-mediated decrease in CRF-R1 mRNA stability. Future studies to either delete or mutate this site are needed to test this hypothesis.

The observed decrease in CRF-R1 mRNA seems to be transient, with levels returning to basal after 24 hr. Vig *et al.* (16) recently reported that pretreatment of AtT-20 cells with the glucocorticoid analogue triamcinolone acetonide also results in a rapid decrease in glucocorticoid-receptor mRNA, which correlates well with previous reports of the autoregulation of this receptor (39, 40). Thus, down-regulation of the glucocorticoid receptor itself could explain the transient nature of the dexamethasone-induced down-regulation of CRF-R1 mRNA found in AtT-20 cells in this study. It might therefore be expected that the decrease in POMC mRNA would also be reversed. However, down-regulation of POMC mRNA may require less of the glucocorticoid receptor for maximal effect. Alternatively, given the much slower onset of the decrease, desensitization of the mechanisms involved might take longer than the 24 hr examined in this study.

In summary, we have demonstrated that CRF-R1 mRNA is down-regulated after glucocorticoid-receptor activation in the pituitary-derived AtT-20 cell line. The mechanism occurs via regulation of both CRF-R1 mRNA stability and gene transcription and requires *de novo* protein synthesis. Given the important role of glucocorticoids in the regulation of a number of *in vivo* systems, further characterization of the molecular mechanisms involved in the regulation of CRF-R1 expression may contribute to a better understanding of the actions of the steroid receptors.





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