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Differential Regulation of Corticotropin-Releasing Factor$_1$ Receptor Expression by Stress and Agonist Treatments in Brain and Cultured Cells

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SUMMARY

Corticotropin-releasing factor (CRF) is known to play a major role in coordinating neuroendocrine and behavioral responses to stress. We demonstrate that expression of the CRF$_1$ receptor (CRF-R1) is regulated by stress in the brain and by agonist treatments in cultured cells. Expression of CRF-R1 mRNA was decreased in the frontal cortex but increased in the hippocampus by chronic unpredictable stress. Chronic corticosterone administration did not influence levels of CRF-R1 mRNA in either region, suggesting that regulation of CRF-R1 expression is mediated by CRF itself or by another stress-related factor. Differential regulation of CRF-R1 mRNA by agonist treatment was also observed in two cultured cell lines. In CATH.a cells, a neuron-derived cell line, incubation with CRF decreased levels of CRF-R1 mRNA, whereas in AtT-20 cells, a pituitary-derived cell line, agonist (CRF) treatment increased levels of CRF-R1 mRNA. Further studies demonstrated that the observed changes in both cell lines could be accounted for by regulation of CRF-R1 gene transcription and not by altered mRNA stability. Furthermore, agonist-induced down-regulation of CRF-R1 transcription rate in CATH.a cells was found to be dependent on *de novo* protein synthesis, suggesting the involvement of an inducible repressor. The results show that different cell types show differential transcriptional regulation of the CRF-R1, which could explain the region-specific regulation of receptor expression in the brain.

CRF is the predominant chemical messenger mediating hypothalamic regulation of the pituitary-adrenal axis in response to stress. In addition, CRF is widely distributed in the brain and is thought to coordinate behavioral, neuroendocrine, and autonomic responses to stress (1–7). Expression of CRF, in turn, is differentially regulated in the brain by stress: CRF expression is increased in the hypothalamus, decreased in the olfactory bulb, and not regulated in the cerebral cortex or brainstem in response to stress (3, 4). Furthermore, alterations of the CRF system have been implicated in certain psychiatric illnesses that are precipitated or exacerbated by stress, most notably, depression and anxiety (8–10).

Although numerous studies have examined the regulation of CRF, there is little known about the mechanisms controlling the expression of CRF receptors in brain. CRF receptors belong to the G protein-coupled receptor superfamily, and recent studies have identified two CRF receptor subtypes (11–13). Both receptor subtypes are expressed in the brain, although CRF-R1 is more widely distributed in the central nervous system, whereas CRF-R2 is also expressed in peripheral tissues, including the cardiovascular system. CRF-R1 and CRF-R2 are both positively coupled to the cAMP second messenger system, and CRF stimulation of adenylyl cyclase activity has been observed in the brain (14), in pituitary cell cultures (15), and in the AtT-20 mouse pituitary tumor cell line (16).

Levels of CRF receptor binding in pituitary or AtT-20 cells are consistently reported to be decreased by stress, agonist (CRF), or corticosterone treatments (17–24), but expression of CRF receptors in the brain seems to be relatively resistant to these treatments. Some studies have failed to show alterations in levels of CRF receptor ligand binding in the brain by chronic stress or corticosterone treatments (17, 19, 23), whereas others have demonstrated that levels of CRF receptors in the brain are decreased by repeated stress (21) or intracisternal administration of CRF (20). In addition, acute stress is reported to transiently increase the expression of CRF-R1 mRNA in the hypothalamus but not in any other

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ABBREVIATIONS: CRF, corticotropin-releasing factor; CRF-R1, corticotropin-releasing factor$_1$ receptor; CREB, cAMP response element-binding protein; CRE, cAMP response element; ICER, immediate cAMP early repressor; bp, base-pair(s); PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

brain region studied (25). These findings suggest that additional adaptations or different regulatory mechanisms may account for the region-specific regulation of CRF receptor expression. In the current study, we investigated these possibilities by examining the influence of acute and chronic stress on the expression of CRF-R1 mRNA in different brain regions and by determining the molecular mechanisms that underlie regulation of CRF-R1 mRNA in two cultured cell lines.

Materials and Methods

Animals and treatment paradigms. Male Sprague-Dawley rats (150–170 g) were used for all experiments. The unpredictable stress paradigm is designed so that the animals do not habituate to the same stressor (26). Elevated blood corticosterone levels have previously been reported using a similar unpredictable paradigm, thus providing a physiological indicator that the rats are being stressed using this method (27). In the current study, rats were subjected for 10 days to two of eight randomly chosen stressors each day according to the following regimen (28): day 1, cage movement on a rotating orbital shaker for 50 min, forced swim in a plastic barrel containing water at 18–22° for 4 min; day 2, cold isolation stress at 5° for 60 min, lights on between 7 p.m. and 7 a.m.; day 3, lights off between 12 p.m. and 3 p.m. followed by cold isolation for 15 min; day 4, cage movement for 50 min followed by food and water deprivation overnight; day 5, forced swim for 3 min, followed by social isolation in individual cages overnight; day 6, forced swim for 2 min, restraint stress for 60 min; day 7, restraint stress for 60 min, lights off for 2 hr; day 8, lights on and food and water deprivation overnight; day 9, cage movement for 20 min, followed by lights on overnight; and day 10, social isolation and food and water deprivation overnight.

Additional experiments were carried out with animals subjected to only 3 days of the above stressors or alternatively 1 or 10 days of restraint stress (45 min/day). For corticosterone treatments, sustained-release pellets (100 mg; Innovative Research of America, Toledo, OH) were implanted subcutaneously. The rats were killed by decapitation the morning after the last stressor or day 7 of corticosterone treatment. All animal use procedures were in strict accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" and approved by the Yale Animal Care and Use Committee.

Cell culture. CATH.a cells were grown in Petri dishes (100 × 20 mm) in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 5% fetal bovine serum, 8% horse serum, and 100 units/ml penicillin/streptomycin (all GIBCO). AtT-20 cells were also grown in Petri dishes (100 × 20 mm) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. Both cell lines were split at a ratio of 1:5 and grown for 4–5 days. Fresh media containing CRF (1 μM; Dr. Jean Rivier, Salk Institute, San Diego, CA) or forskolin (5 μM; Sigma Chemical, St. Louis, MO) were then added for the times indicated. For mRNA stability analysis, the cells were further incubated with the transcription inhibitor actinomycin D (2 μg/ml; GIBCO) for ≤4 hr. The influence of protein synthesis inhibition was determined by the addition of cycloheximide (100 μg/ml) at 2 hr before the addition of the stimulators: cycloheximide addition resulted 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. Serum was maintained in the medium throughout all experimental procedures.

RNase protection analysis. RNA was prepared through homogenization of brain sections or cells in 4 M guanidine thiocyanate/25 mM sodium acetate buffer containing 0.5% 2-mercaptoethanol and then 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, and the concentration was determined by spectrophotometry. The riboprobes were prepared by PCR amplification of a 425-bp fragment of the mouse CRF-R1 (corresponding to bp 614-1039) from 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 described previously (29). This technique was also used to prepare a rat CRF-R1 riboprobe (a 335-bp fragment isolated from rat cerebral cortex cDNA, corresponding to 622–957 bp). For RNase protection analysis (29, 30), 30 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-Mannheim) and SDS for an additional 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 described previously (29, 30). The cells were incubated in the presence or absence of 1 μM CRF or 5 μM forskolin for the times indicated, followed by the addition of the transcription inhibitor, actinomycin D (2 μg/ml). Cells were harvested at different time periods (1–4 hr); the RNA was isolated; and CRF-R1 mRNA was determined by RNase protection assay.

Nuclear run-on analysis. Nuclei from cells treated with vehicle, 1 μM CRF, or 5 μM forskolin were isolated by Dounce homogenization, and nuclear run-on analysis was performed as described previously (29, 30). Nuclei from control and CRF-treated AtT-20 cells were isolated according to the modified method of Kiely *et al.* (31). 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 20 units of DNase 1 (Stratagene, La Jolla, CA) and 4.6 μl of 100 mM $CaCl_2$, the nuclei were incubated for an additional 10 min, and newly transcribed RNA was extracted according to the method of Greenberg and Bender (32). The radiolabeled RNA was denatured and hybridized to CRF-R1, vector, or cyclophilin cDNA immobilized on nylon membranes (~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% polyvinylpyrrolidine, 2% bovine serum albumin), 4 mg of 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.

Adenylyl cyclase assays. Cell particulate fractions were assayed for adenylyl cyclase activity using a modified version of a method described previously (33). Briefly, 10–20 μg of particulate protein was homogenized in a total volume of 100 μl containing 20 mM Tris·HCl, pH 7.4, 1 mM EGTA, and 1 mM dithiothreitol and centrifuged at 12,000 × *g* for 10 min, and the pellets were resuspended in 100 μl of 10 mM Tris·HCl, pH 7.4. Twenty-five microliters of the membrane extract was then added to glass tubes containing 25 μl of assay buffer (0.4 mM EGTA, 4 mM dithiothreitol, 200 mM triethanolamine HCl, pH 7.4), 10 μl of GTP (10 μM), and 30 μl of CRF (1 μM) or vasoactive intestinal peptide (1 μM). The tubes were incubated for 12 min in a 37° shaking water bath; then, 20 μl of reaction mixture {2 mM $MgCl_2$, 0.1 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphokinase, 0.2% bovine serum albumin, 0.05 mM ATP, 0.05 mM [^{32}P]ATP (1 μCi)} was added, and the tubes were incubated for an additional 12 min. The reaction was terminated by the addition of 800 μl of stop mix (0.10% SDS, 0.10 mM cAMP) and analyzed for ^{32}P-labeled cAMP by Dowex/alumina chromatography.

Data analysis. All values are given as mean ± standard error. In most cases, significance testing was carried out by use of a Student's *t* test. For some experiments, the χ^2 test was used for analysis of multiple experiments with different base-line values. Significance was considered at a level of $p < 0.05$.

Results

Regulation of CRF-R1 mRNA in the brain by stress. To investigate the effects of stress on CRF-R1 mRNA, rats were subjected to repeated unpredictable stress or repeated restraint stress. The unpredictable stress paradigm was chosen to reduce habituation of the rats to the applied stressor (27). CRF-R1 mRNA levels in the frontal cortex and hippocampus were measured by RNase protection assay using a riboprobe derived from a 335-bp fragment of the rat CRF-R1 cDNA (Fig. 1). CRF-R1 mRNA levels were significantly decreased in the frontal cortex after 10 days of repeated unpredictable stress (Fig. 2). This effect was dependent on repeated stress because 1 day of restraint stress (97 ± 3% of control, mean ± standard error; four individual animals; data not shown in Fig. 2) or 3 days of unpredictable stress (Fig. 2) did not significantly influence levels of CRF-R1 mRNA. Furthermore, 10 days of repeated restraint stress did not significantly influence the expression of CRF-R1 mRNA in frontal cortex (90 ± 4% of control; six individual animals; data not shown in Fig. 2). In contrast to frontal cortex, levels of CRF-R1 mRNA in hippocampus were significantly increased after 10 days of repeated unpredictable stress but not after 1 day of restraint stress (Fig. 2).

To determine whether the observed changes were due to stress-induced elevation of glucocorticoids, rats were treated with corticosterone. Corticosterone treatment (7 days) did not significantly influence levels of CRF-R1 mRNA in either frontal cortex or hippocampus (89 ± 1% and 102 ± 18% of control, respectively; five or six individual animals).

Regulation of CRF-R1 mRNA in cultured cells by agonist treatment. One potential mechanism by which stress might lead to changes in CRF-R1 mRNA is via increased production of CRF. To examine this possibility, we determined the influence of CRF treatment on expression of CRF-R1 mRNA in two cultured cell lines. CATH.a and AtT-20 cell lines are neuron and pituitary derived, respectively, and both express CRF receptors (34, 35). Functional coupling of CRF receptors to adenylyl cyclase in both cell lines was first demonstrated. Incubation of particulate fractions from either cell line with CRF results in a dose-dependent stimulation of adenylyl cyclase activity. The maximum response to CRF was greater in the CATH.a cells than in AtT-20 cells (560 ± 47% and 292 ± 114% of base-line, respectively; three separate determinations), but the EC_{50} values were similar in the two cell lines (0.23 ± 0.09 and 0.29 ± 0.11 μM, respectively; three separate determinations).

Levels of CRF-R1 mRNA were measured in the CATH.a and AtT-20 cells by RNase protection analysis using a 421-bp

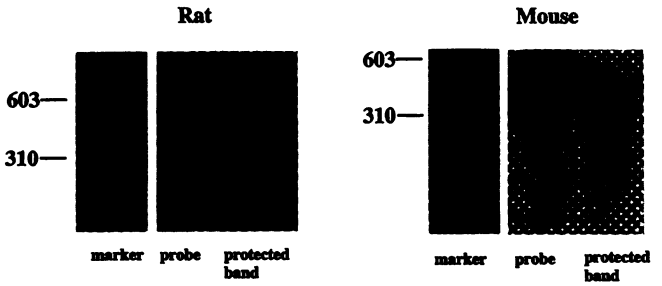

Fig. 1. RNase protection analysis of CRF-R1 mRNA. Rat or mouse CRF-R1 riboprobes were labeled and hybridized with total RNA extracted from either rat frontal cortex or CATH.a cells, a cell line derived from the mouse. After RNase treatment, the protected hybrids were subjected to gel electrophoresis followed by autoradiography as described in Materials and Methods. Also shown are the unhybridized ^{32}P-labeled riboprobes alone and DNA markers.

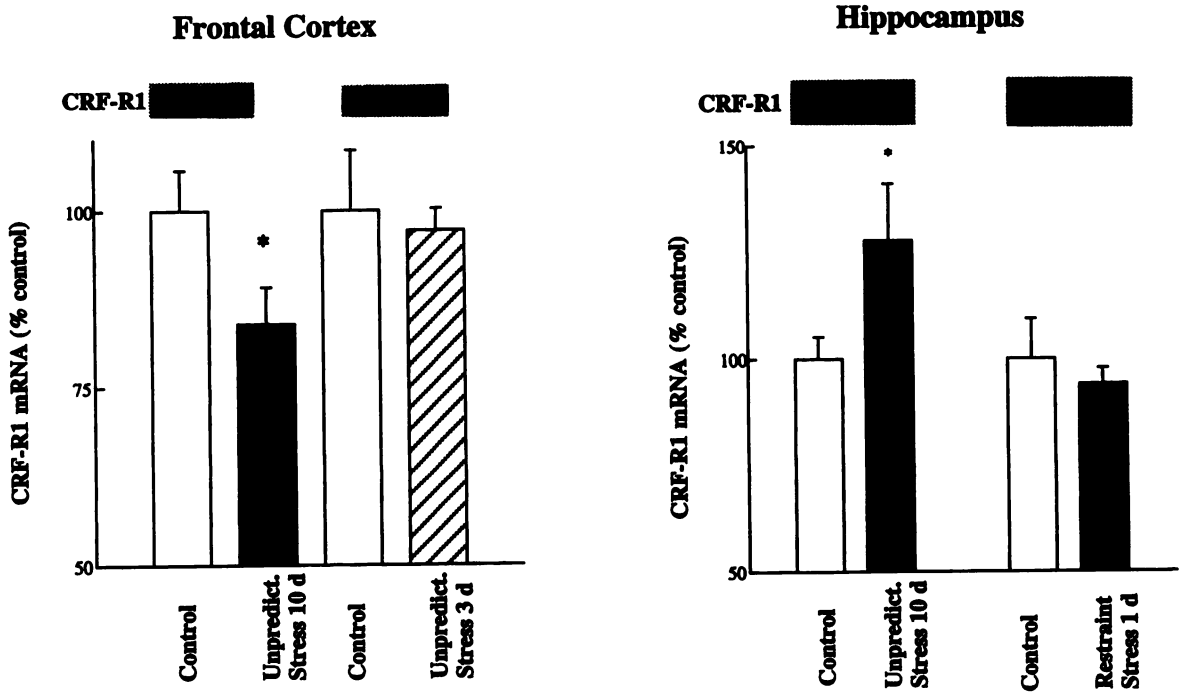

Fig. 2. Regulation of CRF-R1 mRNA in the brain by stress. Rats were subjected to repeated unpredictable stress for 3 or 10 days (*d*) or restraint stress for 1 day as indicated. Levels of CRF-R1 mRNA in frontal cortex and hippocampus were determined by RNase protection analysis as described in Materials and Methods. *Autoradiograms* are representative of the observed changes. Levels of CRF-R1 mRNA were quantified by densitometry. Results are presented as percentage of control and are mean ± standard error of data from 10–12 individual animals. *, $p < 0.05$ compared with control (Student's *t* test).

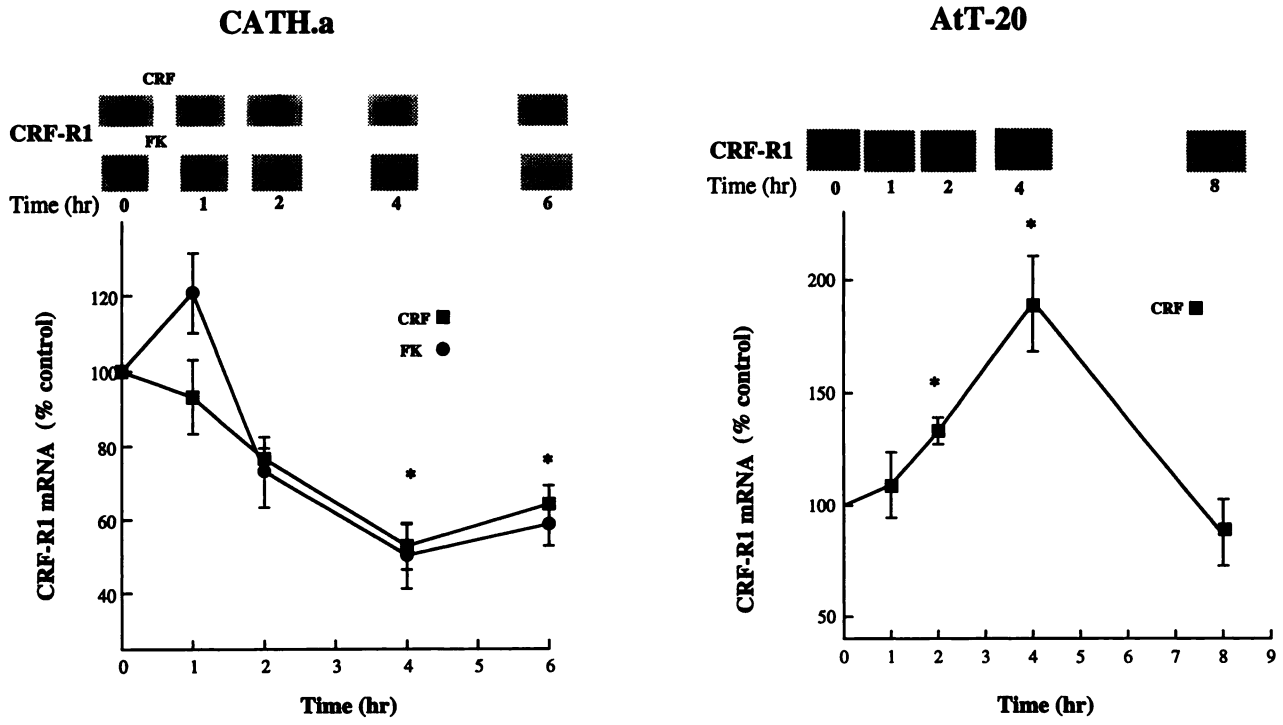

Fig. 3. Regulation of CRF-R1 mRNA in cultured cells. CATH.a and AtT-20 cells were incubated with CRF (1 μM CATH.a, 100 nM AtT-20; ■) or forskolin (*FK*) (5 μM; ●) for the time indicated. RNA was then extracted, and levels of CRF-R1 mRNA were determined by RNase protection analysis. *Autoradiograms* are representative of each treatment condition. Levels of CRF-R1 mRNA were quantified by densitometry. Results are expressed as percentage of control and are mean ± standard error of three to five separate determinations. *, $p < 0.05$ compared with control.

antisense riboprobe isolated by PCR of CATH.a cell cDNA (Fig. 1); the rat probe could not be used because both cell lines are derived from the mouse. Sequence analysis demonstrated that the PCR clone was a homologue (95% identity at the amino acid level) of the rat CRF-R1 (data not shown).

Incubation of CATH.a cells with CRF (1 μM) resulted in a time-dependent decrease in levels of CRF-R1 mRNA that was maximal after 4 hr (Fig. 3) and was not decreased further after 24 hr of CRF treatment (54 ± 15% of control; four separate determinations). This relatively high concentration of CRF was used to avoid any potential problems arising from breakdown of the peptide after long periods in culture. However, the decrease was also observed in preliminary experiments using lower, more physiological concentrations (10 nM) of CRF (52 ± 12% of control; two separate determinations). Similar changes in receptor mRNA were observed after the addition of forskolin, with the maximal change in receptor mRNA levels again occurring after 4 hr (Fig. 3). In contrast, incubation of AtT-20 cells with CRF (100 nM) resulted in a time-dependent increase in CRF-R1 mRNA levels that was also maximal at 4 hr. Levels of CRF-R1 mRNA subsequently returned to base-line after 8 hr of agonist treatment (Fig. 3). However, it should be noted that the apparent reversal of the effect might be a consequence of degradation of CRF, which can be enhanced in the presence of serum.

Analysis of CRF-R1 mRNA stability and gene transcription rate. The stability of CRF-R1 mRNA was examined by determining the rate of mRNA decay after the addition of the transcription inhibitor actinomycin D (Fig. 4). In CATH.a cells, the half-life of CRF-R1 mRNA was determined to be 122 ± 9 min (eight separate determinations), which is similar to that reported for β-adrenergic receptors in other cell lines (29–31). Treatment with CRF did not decrease this half-life, as would be expected if the decrease in levels of CRF-R1 mRNA were due to a change in mRNA stability. Rather, CRF as well as forskolin increased CRF-R1 mRNA half-life (204 ± 10 and 182 ± 22 min; four separate determinations; $p < 0.05$), suggesting that CRF-R1 mRNA stability is increased by agonist treatment. The half-life of CRF-R1 mRNA in AtT-20 cells was similar to that in CATH.a cells, and incubation with CRF resulted a small, nonsignificant increase (162 ± 6 and 204 ± 24 min, respectively; three separate determinations). This could contribute to the elevation of receptor mRNA resulting from agonist treatment but does not seem to be sufficient to account for the observed increase.

These findings suggest that regulation of CRF-R1 mRNA occurs via regulation of gene transcription rather than mRNA stability. This possibility was supported by nuclear run-on studies, which directly measure the rate of CRF-R1 gene transcription. Incubation of CATH.a cells with CRF for 4 hr significantly decreased CRF-R1 transcription rate, whereas incubation of AtT-20 cells with agonist increased the rate of receptor transcription (Fig. 5). In addition, incubation of CATH.a cells with forskolin resulted in a similar down-regulation of CRF-R1 transcription. These findings demonstrate that changes in gene transcription could account for the regulation of CRF-R1 mRNA observed in these two cell lines.

To determine whether there is a requirement for *de novo* protein synthesis in the regulation of CRF-R1 mRNA, the cells were pretreated with cycloheximide, an inhibitor of protein synthesis. Incubation of CATH.a cells with cycloheximide alone significantly increased CRF-R1 mRNA levels

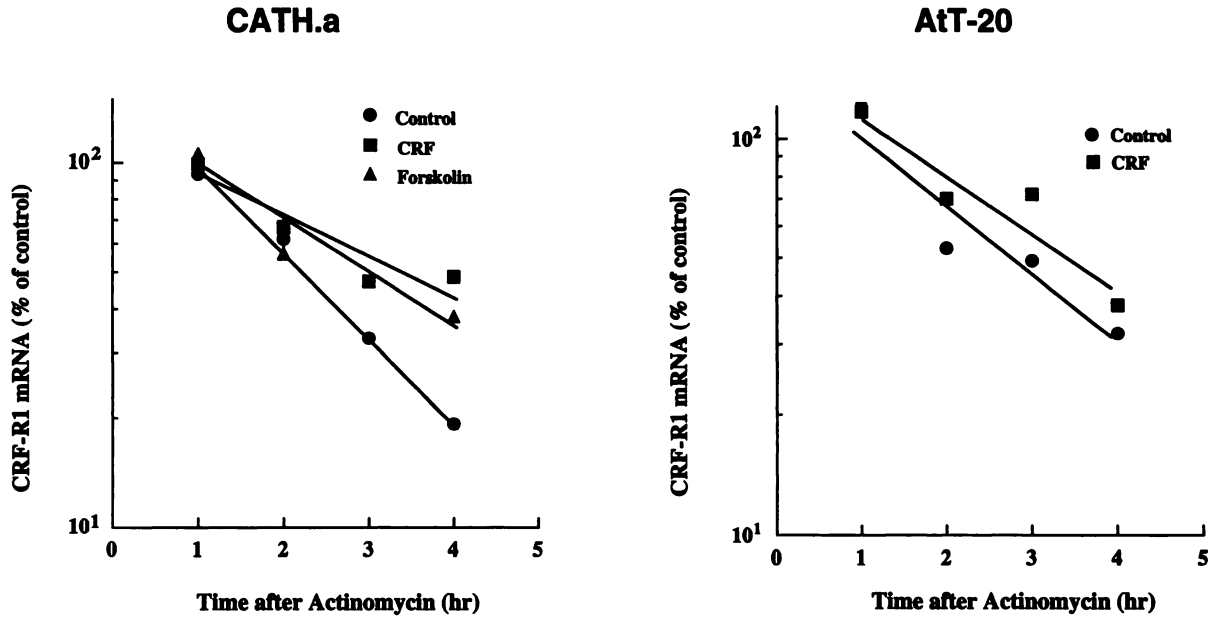

Fig. 4. Determination of CRF-R1 mRNA stability. CATH.a and AtT-20 cells were incubated in the absence (●) or presence of CRF (1 μM CATH.a, 100 nM AtT-20; ■) or forskolin (5 μM; ▲) for 4 or 3 hr, respectively, followed by the addition of actinomycin D. The cells were then harvested at different time intervals over a 4-hr period; RNA was extracted; and CRF-R1 mRNA levels were determined by RNase protection analysis. *Autoradiograms* are representative of each condition. Levels of CRF-R1 mRNA were quantified by densitometry. Results are expressed as percentage of control and plotted on a log-versus-time scale. *Points,* means ± standard error of four and two separate determinations for CATH.a and AtT-20 cells, respectively.

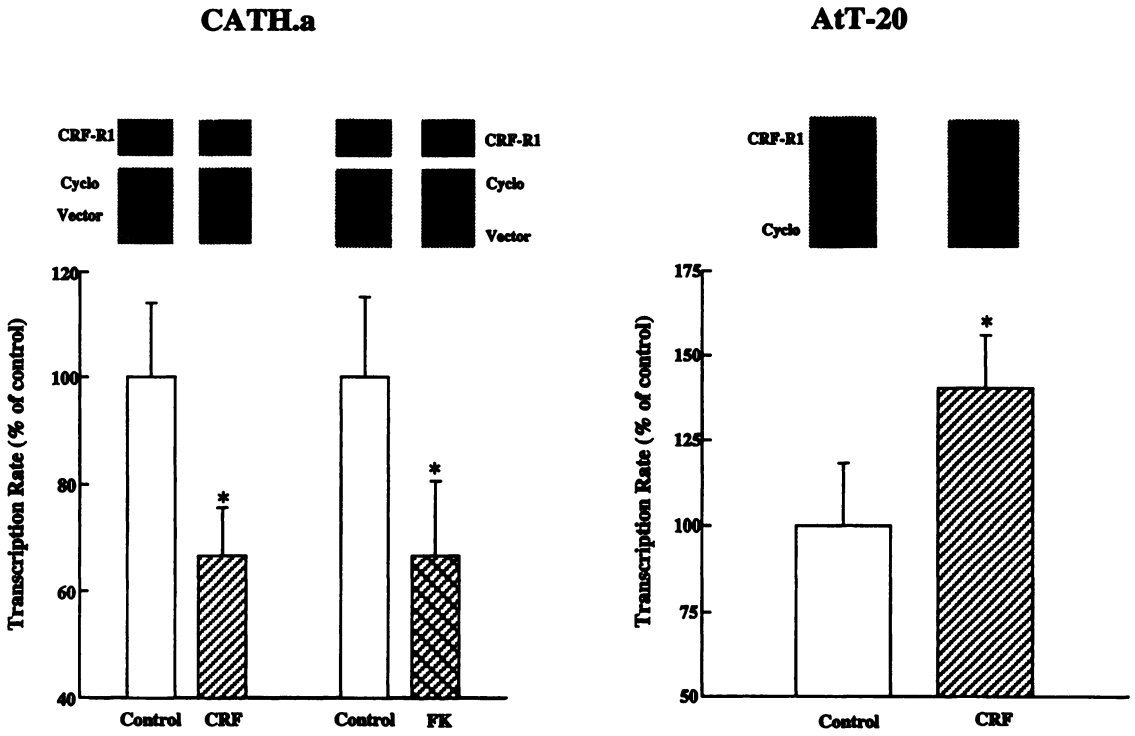

Fig. 5. Determination of CRF-R1 gene transcription rate. CATH.a and AtT-20 cells were incubated in the absence or presence of CRF (1 μM CATH.a, 100 nM AtT-20) or forskolin (*FK*) (5 μM) for 4 or 3 hr, respectively. Cell nuclei were isolated, and transcription elongation was allowed to continue in the presence of [^{32}P]UTP with unlabeled ATP, CTP, and GTP. The radiolabeled nascent RNA was then isolated and hybridized to CRF-R1, cyclophilin (*Cyclo*), and vector cDNA (5 μg), which were immobilized on nylon filters. The labeled filters were then washed and subjected to autoradiography. *Autoradiograms* are representative for each condition. The level of radioactivity in each band was quantified by densitometry. Results are presented as percentage of control and are mean ± standard error of three or four separate determinations. *, $p < 0.05$ compared with control (χ^2 test).

(178 ± 21% of control; three separate determinations; $p < 0.05$). Furthermore, cycloheximide pretreatment of CATH.a cells completely blocked the CRF-induced down-regulation of CRF-R1 mRNA, suggesting that this effect is mediated by induction of a repressor of gene transcription (Fig. 6). In AtT-20 cells, cycloheximide treatment also increased levels of

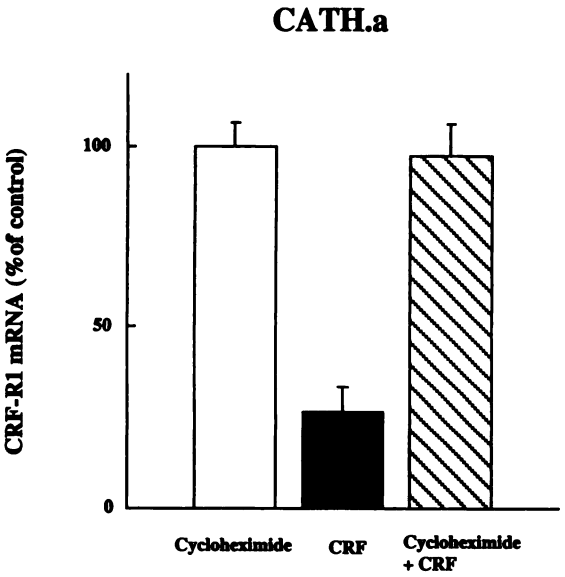

Fig. 6. Influence of cycloheximide on agonist regulation of CRF-R1 mRNA. Cells were pretreated with cycloheximide for 2 hr followed by CRF (1 μM) or vehicle for 4 hr; RNA was then extracted, and CRF-R1 mRNA levels were determined by RNase protection analysis. Results are percentage of cycloheximide. *Bars*, mean ± standard error of five separate determinations. *, $p < 0.05$ compared with control.

CRF-R1 mRNA (224 ± 30% of control; three separate determinations; $p < 0.05$). This effect was not additive with the elevation of CRF-R1 mRNA levels that result from CRF treatment (not shown).

Discussion

Although numerous studies have demonstrated that levels of CRF are regulated by stress and that CRF plays a significant role in the endocrine and behavioral responses to stress (see introductory paragraph), much less is known about the regulation of CRF receptors by chronic stress in the brain. The current study demonstrates that chronic unpredictable stress regulates the expression of CRF-R1 mRNA in frontal cortex and hippocampus. Furthermore, the results demonstrate that expression of CRF-R1 mRNA is differentially regulated in these two regions, with a decrease observed in frontal cortex and an increase in hippocampus. In contrast, repeated exposure to the same stress (i.e., restraint) did not influence expression of CRF-R1 mRNA in frontal cortex. Down-regulation of CRF-R1 expression in frontal cortex may represent a compensatory adaptation to chronic stress, whereas up-regulation of receptor mRNA in hippocampus may represent a sensitization process that could increase subsequent responses to stress that are mediated by this brain region. A recent study demonstrated that acute stress increases CRF-R1 mRNA in hypothalamus, an effect that may also enhance the CRF-responsiveness of this brain region (25). These findings indicate that CRF-mediated responses may be either decreased or increased by chronic unpredictable stress, depending on the brain region involved.

Although a few studies have reported that stress or CRF treatments decrease CRF ligand binding and function in the brain (20, 21), other studies have not reported changes in levels of CRF receptor density (17, 19, 23). This is surprising because CRF receptor density in pituitary is sensitive to regulation by stress, agonist, or glucocorticoid treatments (17–23). One possibility is that there may be additional adaptive mechanisms that counteract the regulation of CRF receptor expression in the brain. This possibility is supported by the results of the current study demonstrating that levels of CRF-R1 mRNA in frontal cortex are regulated by chronic unpredictable stress but not by chronic restraint stress. This could result from habituation to the same stress on repeated exposure and could account for the lack of regulation observed in most previous studies. However, the unpredictable stress paradigm consists of a number of different stressors, any one of which might be responsible for the observed changes. Thus, general stress alone might be insufficient to alter CRF receptor expression in a particular brain area, although an individual stressor could be effective on its own.

Another possibility is that expression of CRF receptors in the brain may be under the control of different regulatory mechanisms than those in the pituitary. This hypothesis is supported by previous reports that corticosterone regulates the expression of CRF receptor ligand binding in the pituitary but not in the brain (17, 18) and by the results of the current study that corticosterone treatment does not influence levels of CRF-R1 mRNA in the brain, at least not under the conditions used. However, this does not rule out the possibility of transient changes, which are no longer present after chronic glucocorticoid treatment, or dose-dependent effects. An alternative hypothesis is that stress-induced increases in CRF or some other factor might mediate regulation of CRF-R1 mRNA expression. Previous reports have demonstrated that exposure to CRF down-regulates levels of CRF receptor ligand binding in the brain and pituitary; however, the relevance of these data must be viewed with some caution given the lack of evidence for correlation between CRF receptor binding and CRF-R1 mRNA. Intracisternal administration of CRF decreases levels of CRF receptor binding in the amygdala (20), and incubation of cerebral cortical primary cultures with CRF decreases levels of CRF receptor binding (36). This raises the possibility that the down-regulation of CRF-R1 mRNA in frontal cortex is an adaptive response to elevated levels of CRF. Indeed, stress is reported to influence levels of CRF in several brain regions, including cerebral the cortex and hippocampus (37, 38). A similar mechanism could account for the down-regulation of CRF receptor binding in the frontal cortex of suicide victims (39) and the decreased CRF responsiveness of locus ceruleus neurons in animals exposed to stress (40). In contrast, the mechanisms underlying the increase in levels of CRF-R1 mRNA in the hippocampus in response to chronic stress observed in the current study and in the hypothalamus in response to acute stress (25) are more difficult to understand. Rivest *et al.* (25) suggested that this may represent a positive feedback loop that enhances the responsiveness of the hypothalamus to subsequent exposure to stress; a similar regulatory loop may occur in the hippocampus.

These *in vivo* studies demonstrate that expression of CRF-R1 in the brain is indeed regulated by stress, but little is known about the cellular and molecular mechanisms that underlie the expression of CRF receptors or neuropeptide receptors in general. To more directly study these mechanisms, we used two cell culture lines: CATH.a and AtT-20. These cell lines express CRF receptors that are functionally coupled to the cAMP second messenger system (24, 34, 35).

We demonstrated that both cell lines express the CRF-R1 subtype and that CRF treatment differentially regulates CRF-R1 mRNA expression in the two cell lines. This indicates that the AtT-20 and CATH.a cell lines may be useful models for delineating the cellular mechanisms that could explain both the up- and down-regulation of CRF-R1 mRNA observed in different brain regions *in vivo*. Studies of mRNA half-life and nuclear run-on analyses indicate that agonist treatment regulates the expression of CRF-R1 mRNA in both cell lines via altered levels of receptor gene transcription, not mRNA stability. These cell culture findings suggest that regulation of CRF-R1 mRNA in the brain may also be mediated by regulation of receptor gene transcription.

Regulation of CRF-R1 mRNA and gene transcription most likely involves the cAMP system because CRF stimulates cAMP formation in both cell lines as well as in the brain (14, 24, 35). In addition, the action of CRF in CATH.a cells is mimicked by direct activation of the cAMP pathway with forskolin. Many actions of the cAMP system on gene transcription are known to be mediated by CREB, which is activated on phosphorylation by cAMP-dependent protein kinase (41). The activation of CREB by this mechanism is believed to increase the transcriptional activity of many genes (41) and therefore could mediate the observed increase in levels of CRF-R1 mRNA in AtT-20 cells. This could also account for the increase in levels of CRF-R1 mRNA in the hippocampus in the current study, as well as that observed in the hypothalamus in response to acute stress (25). Confirmation of this mechanism must await the characterization of the promotor region of the CRF-R1 gene to determine whether it contains functional CREs.

The mechanism by which the activation of the cAMP system down-regulates CRF-R1 mRNA and gene transcription in CATH.a cells seems to involve induction of a repressor because this effect was completely blocked by inhibition of protein synthesis. A similar mechanism might be responsible for the down-regulation of CRF-R1 mRNA observed in the frontal cortex. Although there are several possible transcriptional repressors that could mediate this effect, the cAMP-inducible transcription factor ICER is a likely candidate. ICER is a member of the cAMP response element modulator family of transcription factors that with the exception of one isoform (cAMP response element modulator τ) act as inhibitors of CREB-mediated gene transcription (42). Indeed, recent studies from our laboratory demonstrate a role for ICER in the regulation of β_1-adrenergic receptor gene transcription (43), a finding that is consistent with the role purported for ICER in the down-regulation of follicle-stimulating hormone and thyroid-stimulating hormone receptors (44, 45). We found that CRF increases the expression of ICER in CATH.a cells with a time course consistent with that for down-regulation of CRF-R1 mRNA.[1] However, CRF is also reported to increase the expression of ICER in AtT-20 cells (Ref. 42 and experiments in our laboratory). This induction of ICER could account for the down-regulation of CRF-R1 mRNA observed after longer periods of agonist treatment. The reason for the initial increase in AtT-20 cells is unclear but may result from a difference in the amount or time course of ICER induction or activation of other transcription factors in this cell line.

[1] P. A. Iredale and R. S. Duman, unpublished observations.

The results of this study demonstrate that expression of CRF-R1 can be either increased or decreased depending on the brain region or cell type examined. Decreased expression of receptors could be viewed as an adaptive response to sustained activation of receptors. Although up-regulation of receptor expression may represent a positive regulatory loop between CRF and neurons expressing CRF receptors, as suggested for the hypothalamus (25), it may also represent a maladaptive sensitization process that may contribute to pathophysiological mechanisms that occur after chronic stress. Altered levels of CRF and its receptors have been reported in stress-related illnesses, such as depression (8–10, 37). Characterization of the molecular mechanisms underlying the regulation of CRF receptor expression by stress should contribute to a better understanding of the role of these receptors in such illnesses and, possibly, to the development of novel therapeutic strategies.

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