Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7104–7108, August 1993
Neurobiology

Corticotropin-releasing factor mRNA in rat thymus and spleen

(corticotropin-releasing factor secretion/neuroimmunomodulation/reverse transcription-PCR)

FRASER AIRD*†, CHARLES V. CLEVENGER‡, MICHAEL B. PRYSTOWSKY‡, AND EVA REDEI*

Departments of *Psychiatry and ‡Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104

Communicated by Eliot Stellar, March 1, 1993

ABSTRACT **Corticotropin-releasing factor (CRF) initiates stress-induced immunosuppression via the hypothalamic–pituitary–adrenal axis. CRF has also been shown to have direct stimulatory and suppressive effects on immune cells. We have previously detected immunoreactive and bioactive CRF in the rat spleen and thymus. To determine if CRF is synthesized in these tissues, we analyzed rat spleen and thymus for the presence of CRF mRNA. RNA was reverse transcribed, and the resulting cDNA was amplified by the polymerase chain reaction with CRF gene-specific oligonucleotide primers. After Southern blotting and hybridization with an internal CRF gene probe, a product of the expected size was detected in the spleen, thymus, and hypothalamus (positive control) but not in liver or kidney (negative controls), indicating that CRF is synthesized in the spleen and thymus. Furthermore, CRF could be secreted from splenic and thymic adherent cells in culture. Secretion increased severalfold in response to nordihydroguaiaretic acid (NDGA), a lipoxygenase pathway inhibitor, whereas interleukin 1 had no effect, suggesting that regulation of CRF secretion may differ from that in the hypothalamus. CRF mRNA was detected in NDGA-stimulated thymic adherent cells and in both control and NDGA-stimulated splenic nonadherent cells. The finding that CRF is synthesized in the spleen and thymus suggests that locally synthesized "immune" CRF, acting as an autocrine or paracrine cytokine, may have direct regulatory effects on immune function.**

The neuroendocrine response to stress is orchestrated by the hypothalamic hormone corticotropin-releasing factor (CRF) (1). In response to stress, this 41-aa peptide is secreted from the hypothalamus and passes via the portal circulation to the anterior pituitary, where it stimulates synthesis and secretion of peptides derived from the proopiomelanocortin gene, including adrenocorticotropin hormone (ACTH) and β-endorphin (2). Stress also results in suppression of immune function (3, 4), and hypothalamic CRF is involved in this response via glucocorticoids secreted from the adrenal gland in response to ACTH (5, 6) or alternatively by adrenal-independent mechanism(s) (7, 8). Secretion of brain CRF is also stimulated by increases in endogenous interleukin 1 (IL-1) in the brain (9, 10), resulting in peripheral immune suppression (11).

Exogenous CRF has also been shown to have direct immunosuppressive effects on immune functions *in vivo* (8) and *in vitro* (12). In contrast, CRF can enhance production of IL-1 and interleukin 2 (IL-2) by monocytes (13), and IL-1 can in turn stimulate the secretion of β-endorphin from lymphocytes (14–16). CRF receptors, similar to those found in the anterior pituitary and the brain, have been identified in resident macrophages of mouse spleen (17), and CRF-like peptide and mRNA have been identified in unfractionated human peripheral leukocytes (18). These observations suggest a complex but important role for CRF in integrating the neuroendocrine and immune responses to stress.

Recent evidence suggests that CRF is released locally in response to inflammation and acts as an autocrine or paracrine cytokine (19, 20). Similarly, CRF may function as a paracrine hormone and neurotransmitter in multiple extrapituitary sites, via specific receptors localized in the brain (21) and peripheral target organs, including adrenals (22), testis (23), and placenta (24). Since CRF peptide synthesis occurs at some of these sites (25, 26), this suggests that CRF could also be synthesized and act locally in lymphoid tissues.

Previous work in our laboratory has demonstrated the presence of immunoreactive and bioactive CRF in the rat thymus (27). In the present study, we use the reverse transcription–polymerase chain reaction (RT-PCR) to demonstrate the presence of CRF mRNA in rat thymus and spleen, indicating that CRF is synthesized in these tissues of the immune system. We also detect CRF mRNA in thymic adherent cells and splenic nonadherent cells *in vitro*. Furthermore, we show that CRF peptide is secreted *in vitro* by thymic and splenic adherent cells and that the regulation of CRF secretion in these cells differs from CRF secretion in the hypothalamus. A preliminary report of this work has been presented.§

MATERIALS AND METHODS

Animals. Rat hypothalamus, thymus, and spleen were from 21-day-old Sprague–Dawley males. Rat liver and kidney were from 120-day-old Sprague–Dawley females. Animals were killed by decapitation. Tissues were excised and either extracted immediately as described below or frozen at −80°C. For cell cultures, thymi and spleens were removed under sterile conditions and collected into sterile phosphate-buffered saline at 4°C.

RNA Isolation. Total RNA was isolated using guanidium thiocyanate extraction and LiCl precipitation as described in Abood *et al.* (28). In some cases RNA was further purified through CsCl as described in ref. 29 to remove contaminating genomic DNA.

PCR Primers. CRF gene-specific oligonucleotide primers were synthesized from the published sequence for the rat CRF gene (25). Two 5′ primers were used: CRF-4 (5′-GAGGTACCTCGCAGAACAAC-3′) from exon 1 and CRF-1 (5′-CCGCAGCCGTTGAATTTCTTG-3′) from exon 2. The 3′ primer was CRF-2 (5′-AGATATCGCTATAAAGACACT-3′) from exon 2. CRF-4 and CRF-2 generate products of 895 bp from mRNA and 1582 bp from genomic DNA,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CRF, corticotropin-releasing factor; ACTH, adrenocorticotropin hormone; IL-1, interleukin 1; IL-2, interleukin 2; RT-PCR, reverse transcription–polymerase chain reaction; NDGA, nordihydroguaiaretic acid; α-MSH, α-melanocyte-stimulating hormone.

†To whom reprint requests should be addressed.

§Aird, F., Prystowsky, M. B. & Redei, E., 21st Annual Meeting of the Society for Neuroscience, November 10–15, 1991, New Orleans, p. 827 (abstr. 327.1).

whereas CRF-1 and CRF-2 generate a 720-bp product from both mRNA and genomic DNA (see Fig. 1). β-Actin gene-specific primers were synthesized from the published sequence of a mouse β-actin cDNA (30). Act-1 (5′ primer = 5′-CTTCTACAATGAGCTGCGTGTGGCC-3′) and Act-2 (3′ primer = 5′-GGAGCAATGATCTTGATCTTCATGG-3′) generate a product of 728 bp.

RT-PCR. The GeneAmp RNA PCR kit (Perkin–Elmer/Cetus) was used to carry out the RT-PCR. Two micrograms of total RNA was reverse transcribed into cDNA in 40 μl containing 1× PCR buffer (50 mM KCl/10 mM Tris·HCl, pH 8.3/0.01% gelatin), 5 mM $MgCl_2$, each dNTP at 1 mM, RNase inhibitor at 1 unit/μl, 5 μM oligo$(dT)_{16}$ primer, and reverse transcriptase at 2.5 units/μl. Reaction mixtures were incubated at 42°C for 45 min, boiled for 5 min, and cooled on ice for 5 min. Half (20 μl) of the resulting cDNA was amplified by PCR in 100 μl containing 1× PCR buffer, 2 mM $MgCl_2$, each dNTP at 0.2 mM, 2.5 units of AmpliTaq DNA polymerase, 1 μM CRF-4 (or CRF-1), and 1 μM CRF-2; the other half was amplified in the same buffer with 1 μM Act-1 and 1 μM Act-2. Samples were overlaid with mineral oil and amplified in a DNA thermal cycler programmed for 94°C for 5 min, 30 cycles of 94°C for 2 min, 55°C for 1.5 min, and 72°C for 3 min, followed by 72°C for 7 min.

Southern Blotting and Hybridization. A 20-μl aliquot of each RT-PCR reaction was electrophoresed on a 1.2% agarose gel, and the DNA was blotted onto a nitrocellulose filter. Filters were probed with a 320-bp *Pst* I fragment subcloned from a rat CRF cDNA (25) (see Fig. 1) or mouse β-actin cDNA (30) ^{32}P-labeled by random primer labeling (31). Filters were hybridized for 16 h at 42°C in 50% (vol/vol) formamide buffer as described in ref. 32, washed for 30 min at room temperature in 2× SSC/0.1% SDS (1× SSC = 0.15 M NaCl/0.015 M Na citrate, pH 7.0), washed for 60 min at 52°C in 0.1× SSC/0.1% SDS, and exposed to Kodak XAR-5 film at room temperature. Densitometry was carried out with an image analyzer and Macintosh-based BRAIN system with gray-scale calibration.

Cell Cultures. In the CRF peptide secretion experiments, thymic and splenic cells were seeded at 10^7 and 5×10^6 cells per ml, respectively, in RPMI 1640 medium containing penicillin at 100 units/ml, streptomycin at 100 μg/ml, fungizone at 0.25 μg/ml, and 10% (vol/vol) fetal calf serum. After 2–3 days of incubation, nonadherent cells were removed. After an additional 3 days of incubation, cells were washed twice with serum-free medium and incubated for 24 h in fresh serum-free medium containing different treatments in triplicate. Supernatants were removed and kept at −70°C until determination of CRF concentration by RIA.

In the CRF mRNA experiments, splenic and thymic cells were seeded at 1.5×10^7 cells per ml. After a 2-h incubation, adherent and nonadherent cells were separated by decanting, and both sets of cells were washed twice in the same medium as for the secretion studies but containing steroid-free fetal calf serum. Thymic and splenic adherent cells were seeded at 10^7 and 5×10^6 cells per ml, respectively, and nonadherent cells from both tissues were seeded at 7×10^6/ml. After 3 days of incubation in steroid-free medium, treatments were added in duplicate, and cells were incubated for a further 24 h.

CRF RIA. CRF-like immunoreactivity in tissues was measured in the supernatant of the first RNA precipitation (28) as described (27). CRF-like immunoreactivity in the cell culture medium was measured as described (33) using an antiserum against CRF (human, rat; Peninsula Laboratories). The assay sensitivity was 2–4 pg per tube. The intra- and interassay coefficients of variation were 3.5 and 8%, respectively, at 50% binding.

The CRF secretion data were analyzed statistically using one-way ANOVA followed by Student's *t* test. Statistical significance was considered at $P < 0.05$ after correction for multiple comparisons.

RESULTS

Detection of Thymic and Splenic CRF mRNA. Various tissues from rat were analyzed by RT-PCR for the presence of CRF mRNA using rat CRF gene-specific oligonucleotide primers. Fig. 1 shows a map of the rat CRF gene and mRNA, the position of the CRF gene-specific primers, and the position of the 320-bp hybridization probe used to detect the RT-PCR products. Since primers CRF-4 and CRF-2 span intron 1, the PCR product generated from mRNA (895 bp) is distinguishable from that generated from genomic DNA (1582 bp; i.e., 895 + 687 bp).

RNA from hypothalamus, thymus, and spleen was purified by CsCl centrifugation to remove contaminating genomic DNA. Total RNA (2 μg) from hypothalamus, thymus, spleen, liver, and kidney was reverse transcribed into cDNA. Half of each reaction was amplified by PCR with CRF-4 and CRF-2, whereas the other half was amplified with β-actin gene-specific primers. After gel electrophoresis and transfer to nitrocellulose, the CRF-amplified products were probed with the 320-bp CRF fragment, and the β-actin products were probed with a mouse β-actin cDNA. Hypothalamus, which is known to synthesize CRF, served as a positive control. The expected 895-bp product was detected when hypothalamus RNA was amplified with CRF-4 and CRF-2 (Fig. 2*A*, lane 1). This product was also detected when thymus and spleen

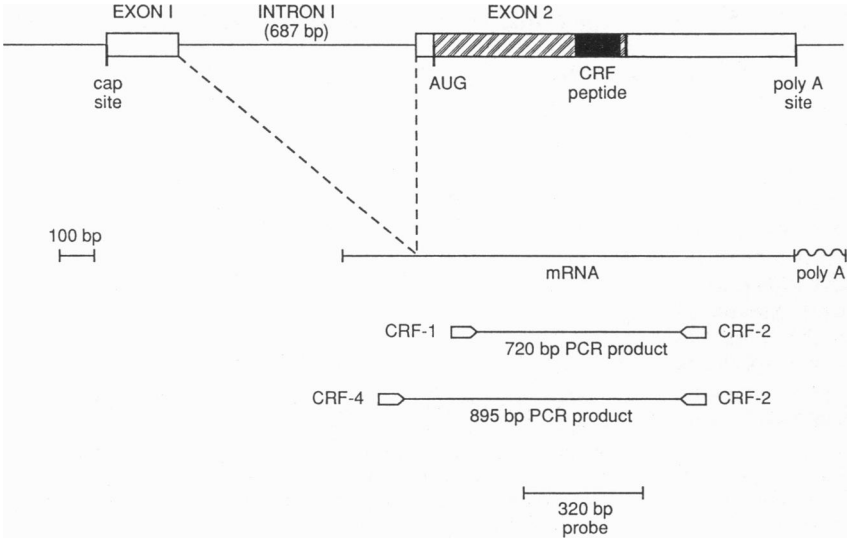

FIG. 1. The rat CRF gene and PCR primers. Exons 1 and 2 are represented as boxes; the hatched and solid regions represent sequences encoding the pro-CRF peptide and the mature 41-aa CRF peptide, respectively (25). The positions of CRF-4, CRF-1, and CRF-2 are shown, along with the CRF-4/CRF-2 895-bp product and the CRF-1/CRF-2 720-bp product generated from CRF mRNA by RT-PCR. CRF-4, nt 110–129 of the rat CRF gene; CRF-1, nt 972–992; CRF-2, nt 1672–1692. Also shown is the 320-bp *Pst* I fragment used to detect CRF-specific PCR products by hybridization.

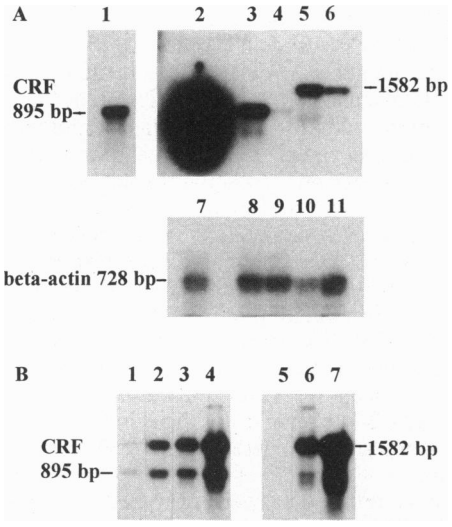

FIG. 2. Detection of CRF mRNA in rat tissues. (*A*) RNA was analyzed by RT-PCR using CRF-4 and CRF-2 (lanes 1–6) and Act-1 and Act-2 (lanes 7–11). Twenty microliters of each RT-PCR reaction mixture was resolved on a 1.2% agarose gel, transferred to nitrocellulose, and hybridized with the 320-bp CRF probe (lanes 1–6) or β-actin cDNA (lanes 7–11). Lanes 1, 2, and 7, hypothalamus; lanes 3 and 8, thymus; lanes 4 and 9, spleen; lanes 5 and 10, liver; lanes 6 and 11, kidney. Lanes 1 and 7–11 were exposed to x-ray film for 5 min, and lanes 2–6 were exposed for 24 h. (*B*) RNA from thymus or spleen (1–8 μg) was analyzed by RT-PCR using CRF-4 and CRF-2. Twenty microliters of each reaction mixture was analyzed by hybridization with the CRF 320-bp probe as described in *A*. Lanes 1–4, 1, 2, 4, and 8 μg, respectively, of thymus total RNA; lanes 5–7, 1, 4, and 8 μg, respectively, of spleen total RNA. Lanes 1–4 were exposed to x-ray film for 6 h, and lanes 5–7 were exposed for 52 h.

RNAs were amplified with these primers (lanes 3 and 4), indicating that CRF mRNA is present in the thymus and spleen. Furthermore, the 1582-bp product was not detected, indicating that genomic DNA was not present in these samples. RT-PCR was also carried out using primers CRF-1 (see Fig. 1) and CRF-2, and the expected 720-bp product was detected in hypothalamus, thymus, and spleen (data not shown). Lane 2 is lane 1 exposed for the same length of time as lanes 3–6 to allow comparison of the relative amount of 895-bp RT-PCR product in hypothalamus, thymus, and spleen. Lane 1 is shown to compare sizes of PCR products in the different tissues. As can be seen in Fig. 2*A* and from densitometry of the autoradiogram (data not shown), significantly more of the 895-bp product was found in the hypothalamus compared to the thymus, and significantly more was found in the thymus compared to the spleen. This experiment has been repeated several times, and these relative levels of the 895-bp product have been observed consistently.

Liver and kidney served as negative controls. The 895-bp product was not detected when RNAs from these tissues were amplified with CRF-4 and CRF-2 (Fig. 2*A*, lanes 5 and 6). The 1582-bp product was detected in liver and kidney, indicating the presence of genomic DNA. Since liver and kidney RNA samples were not purified by CsCl centrifugation, we anticipated that this product would be generated. This demonstrates that CRF-4 and CRF-2 were capable of specific amplification of CRF sequences in the liver and kidney PCRs. Amplification with the β-actin primers (lanes 7–11) demonstrated that all of the RNA samples, including liver and kidney, could be reverse transcribed into cDNA and amplified efficiently by PCR.

In addition to the expected PCR products, a number of bands of unknown origin were detected after amplification with CRF-4 and CRF-2: a 520-bp band in hypothalamus and thymus (Fig. 2*A*, lanes 1 and 3) and a 750-bp band in liver and kidney (lanes 5 and 6). These bands may represent mRNA species that share sequence homology with the CRF gene or may be artifacts due to nonspecific priming during PCR.

In Fig. 2*A*, the intensity of the 895-bp product detected in the spleen was very low compared to that in the hypothalamus. Therefore we repeated the above experiment for thymus and spleen using increasing amounts of total RNA (1–8 μg) for the RT-PCR. As shown in Fig. 2*B*, the intensity of the CRF mRNA-specific 895-bp product increased with increasing amounts of RNA in both the thymus (lanes 1–4) and spleen (lanes 5–7). In this case, RNA preparations were not purified by CsCl centrifugation, and the 895-bp and 1582-bp products were generated from mRNA and genomic DNA, respectively. Fig. 2*B* again demonstrates the significantly higher levels of the 895-bp product generated from the thymus compared to the spleen, since the thymus lanes were exposed to x-ray film for 6 h, whereas the spleen lanes were exposed for 52 h.

CRF Content of Hypothalamic, Thymic, and Splenic Tissue. The CRF peptide content in the supernatant of the first RNA precipitation was 609 ± 28 pg/mg, 8.9 ± 0.7 pg/mg, and 5.2 ± 0.7 pg/mg of wet weight for hypothalamus, thymus, and spleen, respectively, giving ratios of CRF peptide per mg of wet weight for hypothalamus/thymus/spleen of ≈117:1.7:1.

Secretory Response of Thymic and Splenic Adherent Cells. Thymic and splenic adherent cells secreted measurable and nearly equal amounts of CRF in their basal state (Fig. 3). CRF secretion from adherent cells of both spleen and thymus showed minimal changes in response to IL-1 (0.1 and 1 ng/ml), indomethacin (10 and 50 μM), 15-hydroxyeicosatetraenoic acid (500 pg/ml), and leukotriene B_4 (20 pg/ml), which were not significant at $P < 0.05$ (Table 1). Both splenic and thymic adherent cells increased CRF secretion several-fold over baseline in response to different doses of nordihydroguaiaretic acid (NDGA), a lipoxygenase biosynthesis inhibitor (Fig. 3).

CRF mRNA in Adherent and Nonadherent Cells from Thymus and Spleen. Adherent and nonadherent cells from thymus and spleen were analyzed by RT-PCR for the presence of CRF mRNA. In thymic adherent cells, the 895-bp RT-PCR product was not detected in the basal state (Fig. 4, lane 1) but was detected after treatment with 5 and 10 μM NDGA (lanes 2 and 3, respectively). In thymic nonadherent cells, CRF mRNA was not detected in either control or NDGA-treated cells (data not shown). CRF mRNA was detected in splenic nonadherent cells in both the absence (lane 4) and presence of NDGA (5 and 10 μM, lanes 5 and 6, respectively) but was not detected in splenic adherent cells (data not shown). The CRF mRNA levels detected in NDGA-stimulated thymic adherent cells and splenic nonadherent

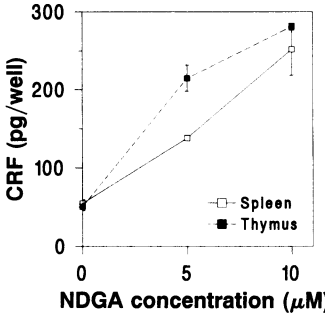

FIG. 3. Concentration-dependent effects of NDGA on CRF secretion. Splenic and thymic adherent cells were incubated for 24 h in the presence or absence of NDGA. Values represent mean ± SEM of two separate experiments, each performed in triplicates. CRF secretion was significantly different from controls at all concentrations of NDGA tested ($P < 0.05$ or lower).

Table 1. Effect of various treatments on CRF secretion in cultured adherent cells from spleen and thymus

Addition	CRF immunoreactivity, pg per well	
	Spleen	Thymus
None (medium)	42.3 ± 9.2	53.1 ± 4.5
IL-1 (0.1 ng/ml)	38.2 ± 4.5	35.3 ± 4.7
IL-1 (1 ng/ml)	40.6 ± 6.2	53.8 ± 8.8
Indomethacin (10 μM)	65.5 ± 3.3	73.2 ± 5.9
15-HETE (500 pg/ml)	74.6 ± 15.9	67.3 ± 8.7
Leukotriene B_4 (20 pg/ml)	21.5 ± 7.5	38.5 ± 6.8

HETE, hydroxyeicosatetraenoic acid. Values are the mean ± SEM (n = 3). Cultured adherent cells were treated for 24 hr. No significant treatment effects were found by multiple comparisons.

cells in culture are comparable, because the thymus lanes were exposed for 2 h and the spleen lanes were exposed for 5 h. This is in contrast to the large difference in CRF mRNA levels observed in thymus and spleen tissues.

These RNA samples were not purified by CsCl centrifugation, as reflected by the presence of the 1582-bp product from genomic DNA in lanes 3–5. In all cases, the expected PCR product was generated using β-actin primers (lanes 7–12), including thymic nonadherent cells and splenic adherent cells (data not shown). This demonstrates that all RNA samples were reverse transcribed into cDNA, which could be amplified efficiently by PCR. Therefore, the absence of CRF mRNA-specific PCR product in control thymic adherent cells, thymic nonadherent cells, and splenic adherent cells was not due to the absence of cDNA.

DISCUSSION

To our knowledge, this report is the first to demonstrate the presence of CRF mRNA in rat thymus and spleen, confirming that CRF is synthesized in these tissues. In addition, this is the first report that CRF can be secreted from thymic and splenic adherent cells in culture. Secretion occurs even in the basal unstimulated state and increases in response to NDGA. We also show that CRF mRNA is present in thymic adherent cells in the presence of NDGA and in splenic nonadherent cells both in the presence and absence of NDGA.

CRF synthesis has been detected in several nonbrain tissues including rat adrenal gland, testis, and pituitary (25).

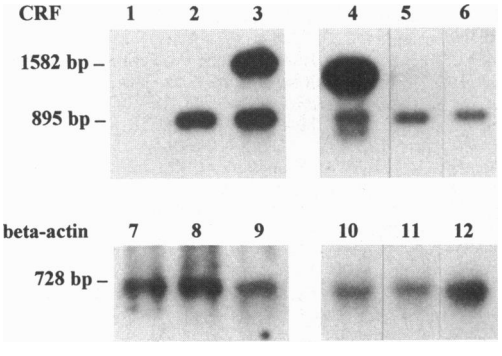

FIG. 4. Effect of NDGA on CRF mRNA levels in thymic adherent cells and splenic nonadherent cells. Thymic adherent cells (lanes 1–3 and 7–9) and splenic nonadherent cells (lanes 4–6 and 10–12) were incubated for 24 h in medium only (lanes 1, 4, 7, and 10) or in the presence of NDGA at 5 μM (lanes 2, 5, 8, and 11) or 10 μM (lanes 3, 6, 9, and 12). RNA was analyzed by RT-PCR using CRF-4 and CRF-2 (lanes 1–6) and Act-1 and Act-2 (lanes 7–12). PCR products were analyzed by hybridization with the 320-bp CRF probe and β-actin cDNA as described in Fig. 2A. Filters were exposed to x-ray film for 2 h at −80°C (lanes 1–3), 5 h at −80°C (lanes 4–6), or 5 min at room temperature (lanes 7–12).

The adrenal and testis transcripts are longer by 0.2 and 0.5 kb, respectively, than the 1.4-kb transcript in rat hypothalamus. CRF mRNA has also been detected in human placenta (26) and peripheral leukocytes (18). A length of 1.7 kb has been reported for the latter, compared to 1.5 kb for the human hypothalamus transcript (18). In this study, the RT-PCR product detected in the thymus and spleen was the same length as that in the hypothalamus, suggesting that the CRF mRNA species in the spleen and thymus are identical to the hypothalamus mRNA. In this analysis, however, differences upstream from the 5′ primer (e.g., in initiation sites) or downstream from the 3′ primer (e.g., in polyadenylylation sites) would not be detected.

Significantly higher levels of CRF mRNA were detected in the thymus compared to the spleen, yet the CRF peptide levels were only 1.7-fold higher in the thymus. This suggests that the splenic CRF transcript may be translated more efficiently than the thymic transcript or that the CRF peptide may be more stable in the spleen compared to the thymus.

Hypothalamic CRF is involved both directly and indirectly in stress-induced immunosuppression (11). However, *in vitro*, CRF directly stimulates lymphocyte proliferation (34), enhances the proliferative response to mitogens, and increases the expression of IL-2 receptor (13, 35). These seemingly contradictory data suggest that CRF might have local direct effects on immune processes that are different from those of hypothalamic CRF. CRF has also been found at peripheral inflammatory sites, where in contrast to its systemic indirect immunosuppressive effects, it acts as an autocrine or paracrine inflammatory cytokine (20). The preliminary data presented here on CRF secretion from adherent splenic and thymic cells indicate that the locally synthesized CRF can be secreted, which provides evidence for a possible direct local effect of CRF on immune processes.

Considering these functional differences found in hypothalamic and immune CRF, it is not surprising that we found the regulation of CRF secretory responses in the spleen or thymus to differ from that of the hypothalamus, despite the apparent similarity of both CRF mRNA and processed CRF peptide in these tissues. A major difference is the lack of responsiveness of thymic or splenic adherent cells to IL-1 stimulation. IL-1 has been shown to stimulate CRF secretion from the hypothalamus directly (10). The IL-1 doses of effective hypothalamic CRF stimulation *in vitro* were between 1 and 50 units/ml (10, 36). Clearly, the IL-1 doses used in this study are higher (100–1000 units/ml); therefore, a possible biphasic effect of IL-1 on CRF secretion from thymic and splenic adherent cells cannot be excluded. Alternatively, the secretory roles of CRF and IL-1 may be reversed in immune cells, since CRF has been shown to stimulate IL-1 production in monocytes (13, 14). Also, increased plasma levels of IL-1 and IL-2 have been reported after intravenous administration of CRF in humans.¶

Another difference between hypothalamic and thymic or splenic CRF secretory responses is the effect of arachidonic acid metabolites on CRF secretion. We have previously shown that blocking the cyclooxygenase and to a lesser degree the lipoxygenase pathway can result in increased secretion of CRF from a hypothalamic preparation (36). In this study, the cyclooxygenase blocker indomethacin was without any significant effect, but the lipoxygenase inhibitor NDGA was a very effective stimulator of splenic and thymic CRF secretion. Further studies should elucidate the tissue-specific interrelationships of CRF and IL-1, the physiological role of CRF stimulation by NDGA, and whether IL-1 is induced in the spleen and thymus by locally synthesized CRF.

¶Schulte, H. M., Monig, H., Bamberger, C. M., Karl, M., Genau, M. & Barth, J., Ninth International Congress of Endocrinology, August 30–September 5, 1992, Nice, France, p. 80 (abstr.).

Although cell types were not determined in this study, it is reasonable to suggest that thymic epithelial cells constitute the majority of thymic adherent cells that express and secrete CRF. Recently, oxytocin and vasopressin mRNA-containing neuroendocrine cells were identified in the thymus, and the epithelial nature of these cells was confirmed (37, 38). Also, our data demonstrating CRF mRNA in the splenic nonadherent cell fraction, consisting mostly of lymphocytes, are consistent with the report that CRF-like mRNA has been found in unstimulated human lymphocytes and neutrophils (18). It is interesting to note that CRF mRNA levels in splenic nonadherent cells were almost comparable with those in NDGA-stimulated thymic adherent cells, whereas CRF mRNA levels were much lower in the spleen compared to the thymus. It is possible that removal of splenic adherent cells removes a negative regulatory factor for CRF mRNA synthesis, which results in enhanced CRF mRNA synthesis in the nonadherent cell fraction. Alternatively, these culture conditions may selectively promote the growth of CRF-synthesizing cells, which results in a greater proportion of CRF mRNA-containing cells in culture than in the intact spleen.

Splenic adherent cells may take up exogenous CRF *in vivo* from the CRF-synthesizing cells of the nonadherent fraction since CRF mRNA was not detected in this cell fraction either in the presence or absence of NDGA. Secretion of this CRF from adherent cells can then be regulated by stimuli such as NDGA. An example of this has been demonstrated in rat and mouse megakaryocytes, which do not synthesize fibrinogen, albumin, or IgG, but take up these proteins from plasma into secretory granules by endocytosis (39).

The regulation of immune CRF and the extent to which CRF participates in mounting an immune response are not known. CRF can induce production of the proopiomelanocortin-derived peptides ACTH and β-endorphin from leukocytes (40) directly or by inducing monocytes to produce IL-1, which in turn activates B lymphocytes to secrete β-endorphin (14). Interestingly, another proopiomelancortin peptide, α-melanocyte-stimulating hormone (α-MSH), seems to play a central and peripheral immunoregulatory role. α-MSH can abolish the central immunosuppressive effects of IL-1 in the brain (41) and the peripheral immunoenhancing ability of IL-1 (42), although contradictory results have been reported (43). It has been suggested that at least some of the immunosuppressive effects of ACTH are in fact due to its conversion to α-MSH by proteolytic cleavage (44). Thus, if α-MSH can antagonize IL-1 activity in the periphery, it could constitute a paracrine negative feedback.

The short half-life of secreted CRF peptide and the probable presence of CRF-binding proteins (45) imply that these direct effects of CRF on immune functions likely involve short-range interactions between target immune cells and locally synthesized CRF. Local synthesis would also ensure higher concentrations of CRF, compared to the very low plasma CRF levels (46) of hypothalamic origin. Site-specific regulation of local CRF secretion could also fine-tune the kinetics of the immune response to stress. Our results suggest that the direct effects of CRF on immune cells are mediated by thymic and splenic CRF, which may act as an autocrine or paracrine hormone, whereas the more long-term effects of stress are mediated by hypothalamic CRF acting via the neuroendocrine system and glucocorticoids.

We thank Li-fang Li for technical assistance in the secretion studies and the CRF assays and Dr. Ildiko Halasz for assistance in the adherent and nonadherent cell culture studies. We also thank Dr. Robert C. Thompson for the rat CRF cDNA clone and initial encouragement. This work was supported by Grant AA 07389 (E.R.) from the National Institute of Alcohol Abuse and Alcoholism. M.B.P. is supported in part by an American Cancer Society Faculty Research Award.

1. Vale, W. W., Spies, J., Rivier, C. & Rivier, J. (1981) *Science* **213,** 1394–1397.
2. Jones, M. T. & Gillham, B. (1988) *Physiol. Rev.* **68,** 743–818.
3. Bateman, A., Singh, A., Kral, T. & Solomon, S. (1989) *Endocr. Rev.* **10,** 92–112.
4. Weiss, J. M., Sundar, S. K., Becker, K. J. & Cierpial, M. A. (1989) *J. Clin. Psychol.* **50,** 43–53.
5. Munck, A., Guyre, P. M. & Holbrook, N. J. (1984) *Endocr. Rev.* **5,** 25–44.
6. Fauci, A. S. (1976) *Clin. Exp. Immunol.* **24,** 54–62.
7. Keller, S. E., Weiss, J. M., Schleifer, S. J., Miller, N. E. & Stein, M. (1983) *Science* **221,** 1301–1304.
8. Jain, R., Zwickler, D., Hollander, C. S., Brand, H., Saperstein, A., Hutchinson, B., Brown, C. & Audhya, T. (1991) *Endocrinology* **128,** 1329–1336.
9. Suda, T., Tozawa, F., Ushiyama, T., Sumimoto, T., Yamada, M. & Demura, H. (1990) *Endocrinology* **126,** 1223–1228.
10. Tsagarakis, S., Gillies, G., Rees, L. H., Besser, M. & Grossman, A. (1989) *Neuroendocrinology* **49,** 98–101.
11. Sundar, S. K., Cierpial, M. A., Kilts, C., Ritchie, J. C. & Weiss, J. M. (1990) *J. Neurosci.* **10,** 3701–3706.
12. Audhya, T., Jain, R. & Hollander, C. S. (1991) *Cell Immunol.* **134,** 77–84.
13. Singh, V. K. & Leu, S. J. (1990) *Neurosci. Lett.* **120,** 151–154.
14. Kavelaars, A., Ballieux, R. E. & Heijnen, C. J. (1989) *J. Immunol.* **142,** 2338–2342.
15. Kavelaars, A., Ballieux, R. E. & Heijnen, C. J. (1990) *Life Sci.* **46,** 1233–1240.
16. Kavelaars, A., Berkenbosch, F., Croiset, G., Ballieux, R. E. & Heijnen, C. J. (1990) *Endocrinology* **126,** 759–764.
17. Webster, E. L., Tracey, D. E., Jutila, M. A., Wolfe, S. A. & De Souza, E. B. (1990) *Endocrinology* **127,** 440–452.
18. Stephanou, A., Jessop, D. S., Knight, R. A. & Lightman, S. L. (1990) *Brain Behav. Immunol.* **4,** 67–73.
19. Hargreaves, K. M., Costello, A. H. & Joris, J. L. (1989) *Neuroendocrinology* **49,** 476–482.
20. Karalis, K., Sano, H., Redwine, J., Listwak, S., Wilder, R. & Chrousos, G. P. (1991) *Science* **254,** 421–423.
21. De Souza, E. B. (1987) *J. Neurosci.* **7,** 88–100.
22. Dave, J. R., Eiden, L. E. & Eskay, R. L. (1985) *Endocrinology* **116,** 2152–2159.
23. Ulisse, S., Fabbri, A. & Dufau, M. L. (1989) *J. Biol. Chem.* **264,** 2156–2163.
24. Petraglia, F., Sawchenko, P. E., Rivier, J. & Vale, W. W. (1987) *Nature (London)* **328,** 717–719.
25. Thompson, R. C., Seasholtz, A. F. & Herbert, E. (1987) *Mol. Endocrinol.* **1,** 363–370.
26. Grino, M., Chrousos, G. P. & Margioris, A. N. (1987) *Biochem. Biophys. Res. Commun.* **148,** 1208–1214.
27. Redei, E. (1992) *Neuroendocrinology* **55,** 115–118.
28. Abood, M. E., Eberwine, J. H., Erdelyi, E. & Evans, C. J. (1990) *Mol. Brain Res.* **8,** 243–248.
29. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
30. Tokunaga, K., Taniguchi, H., Yoda, K., Shimizu, M. & Sakiyama, S. (1986) *Nucleic Acids Res.* **14,** 2829.
31. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
32. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 9.47.
33. Redei, E., Branch, B. J., Gholami, S., Lin, E. Y. R. & Taylor, A. N. (1988) *Endocrinology* **123,** 2736–2743.
34. McGillis, J. P., Park, A., Rubin-Fletter, P., Turek, C., Dallman, M. F. & Payan, D. G. (1989) *J. Neurosci. Res.* **23,** 346–352.
35. Singh, V. K. (1989) *J. Neuroimmunol.* **23,** 257–262.
36. Redei, E., Branch, B. J., McGinnis, R. E. & Taylor, A. N. (1990) *Soc. Neurosci. Abstr.* **16,** 453.
37. Geenen, V., Legros, J. J., Franchimont, P., Defresne, M. P., Boniver, J., Ivell, R. & Richter, D. (1987) *Ann. N.Y. Acad. Sci.* **496,** 56–66.
38. Moll, U. M., Lane, B. L., Robert, F., Geenen, V. & Legros, J. J. (1988) *Histochemistry* **89,** 385–390.
39. Handagama, P., Rappolee, D. A., Werb, Z., Levin, J. & Bainton, D. F. (1990) *J. Clin. Invest.* **86,** 1364–1368.
40. Smith, E. M., Morrill, A. C., Meyer, J. W., III, & Blalock, J. E. (1986) *Nature (London)* **321,** 881–882.
41. Sundar, S. K., Becker, K. J., Cierpial, M. A., Carpenter, M. D., Rankin, L. A., Fleener, S. L., Ritchie, J. C., Simson, P. E. & Weiss, J. M. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 6398–6402.
42. Cannon, J. G., Tatro, J. B., Reichlin, S. & Dinarello, C. A. (1986) *J. Immunol.* **137,** 2232–2236.
43. Daynes, R. A., Robertson, B. A., Cho, B.-H., Burnham, D. K. & Newton, R. (1987) *J. Immunol.* **139,** 103–109.
44. Smith, E. M., Hughes, T. K., Jr., Hashemi, F. & Stefano, G. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 782–786.
45. Potter, E., Behan, D. P., Fischer, W. H., Linton, E. A., Lowry, P. J. & Vale, W. W. (1991) *Nature (London)* **349,** 423–426.
46. Linton, E. A., McLean, C., Kruseman, A. C. N., Tilders, F. J., Van der Veen, E. A. & Lowry, P. J. (1987) *J. Clin. Endocrinol. Metab.* **64,** 1047–1053.