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Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart

(G protein-coupled receptors/HPA axis/cAMP/peptide hormone receptor)

MARILYN PERRIN*†, CYNTHIA DONALDSON*, RUOPING CHEN*, AMY BLOUNT*, TRAVIS BERGGREN*, LOUISE BILEZIKJIAN*, PAUL SAWCHENKO‡, AND WYLIE VALE*

*The Clayton Foundation Laboratories for Peptide Biology and ‡Laboratory of Neuronal Structure and Function, The Salk Institute for Biological Studies, La Jolla, CA 92037

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ABSTRACT **Corticotropin-releasing factor (CRF; corticoliberin) regulates the secretion of corticotropin (ACTH) and β-endorphin and has a broad range of effects on the nervous, endocrine, reproductive, cardiovascular, gastrointestinal, and immune systems. Recently, human, rat, and mouse CRF receptors (CRF-R) have been cloned and functionally and anatomically characterized. We report here the cloning of a second CRF-R cDNA (CRF-RB), which encodes a protein of 431 amino acids, which is 16 amino acids longer and 68% similar to the previously cloned CRF-R, CRF-RA. When transiently expressed in COS-M6 cells, CRF-RB binds CRF with high affinity [K_d = 1.2 (0.57–2.5) nM] and transduces the CRF-stimulated signal of the accumulation of intracellular cAMP, which is inhibited by a CRF antagonist. Comparison of the amino acid sequences of CRF-RB and the previously cloned receptor reveals major differences in the N-terminal domain and in the extracellular loops, whereas the sequences of the intracellular loops are nearly identical. CRF-RB and related transcripts are expressed in the heart, as well as in other tissues, including the gastrointestinal tract, epididymis, and brain.**

Corticotrophin-releasing-factor (CRF; corticoliberin), the 41-amino acid peptide originally isolated from the hypothalamus (1) as the major regulator of corticotropin (ACTH) and β-endorphin secretion by the anterior pituitary, has been shown to be widely distributed in, and to have multiple effects on, a wide variety of tissues (2, 3). Consistent with the broad range of roles proposed for CRF, high-affinity binding sites have been found in pituitary (4), brain (5, 6), adrenals (7), spleen (8), and monocytes (9). Recently, our group (10, 11) and others (12, 13) reported the cloning of CRF receptors (CRF-R), which we now refer to as CRF-RA, from pituitary and brain. These receptors belong to the seven transmembrane domain (TMD) calcitonin/vasoactive intestinal peptide/growth hormone-releasing hormone receptor family. The distribution (14) and functionality of CRF-RA indicated that it satisfied many criteria for a physiologic CRF receptor. In a human Cushing disease tumor cDNA library, we also observed the presence of a splice variant, CRF-RA_2 (10), in which 29 amino acids are inserted into the first intracellular loop.

During the course of the characterization of the mouse gene encoding the CRF-R, we obtained evidence for a related gene, CRF-RB, which we partially sequenced. RNase protection analysis indicated high expression of this gene in the heart. We report here the cloning and characterization of a cDNA from a mouse heart cDNA library encoding a second CRF-R.§

MATERIALS AND METHODS

Phage Library Screening. A 1.2-kb *Pst* I cDNA fragment containing the majority of the coding region of rat CRF-RA was used to screen a mouse genomic library (Stratagene) by standard methods. The filters were washed with 3× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 60°C. Dot blots of phage DNA prepared from hybridization-positive clones were hybridized with the same *Pst* I fragment in QuickHyb solution (Stratagene) and washed under both high- and low-stringency conditions to distinguish clones representing CRF-RA from related receptors. A clone representing a related receptor, designated as CRF-RB, was subcloned into pBluescript II SK (Stratagene) and further characterized by Southern blotting and DNA sequencing.

Approximately 1.2 × 10^6 phage plaques from an amplified oligo(dT)-primed mouse heart cDNA library in the Lambda Zap II vector (Stratagene) were screened by standard methods by hybridization in 20% formamide and final washes in 2× SSC/0.1% SDS at 50°C. The probe was prepared by PCR by using [α-^{32}P]dCTP and the following primers: sense strand, 5′-CTGCATCACCACCATCTTCAACT-3′; and antisense strand, 5′-AGCCACTTGCGCAGGTGCTC-3′. The template used in generating the probe was plasmid DNA corresponding to one exon of CRF-RB extending from amino acid 206 to 246 (see Fig. 1). PCR amplification was carried out for 30 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 3 min) to yield a 120-bp product.

A hybridization-positive plaque was isolated and purified in the next round of plaque hybridization. Helper phage R408 (Bio-Rad) was used for *in vivo* excision of the Lambda Zap II clone. The DNA sequence of both DNA strands of the cloned receptor was determined by the dideoxynucleotide chain-termination method by using the Sequenase kit (United States Biochemical). For transfection studies, the full-length receptor was subcloned into the *Eco*RI restriction site of pcDNA I (Invitrogen).

Alignments of nucleotide or amino acid sequences were done by using the Jotun–Hein method, with the weighted or the PAM250 residue-weight table, respectively.

RNase Protection Assay. The coding region for the mouse receptor corresponding to CRF-RA was cloned by reverse transcription–PCR by using primers based on the published sequence (12) and RNA from AtT-20 cells as the template. Plasmid DNA encoding amino acids 26–106 of mouse CRF-RA (in pCRII; Invitrogen) and 1–132 of mouse CRF-RB (in pcDNA I) was linearized with *Eco*RV and *Bam*HI, respectively, and antisense riboprobes were synthesized by using SP6 RNA polymerase in the presence of [α-^{32}P]UTP. As an internal loading control, the DNA encoding mouse glyceraldehyde-3-phosphate dehydrogenase (in pTRI, Ambion, Austin, TX) was linearized with *Sty* I, and an

Abbreviations: CRF, corticotropin-releasing factor; CRF-R, CRF receptor; TMD, transmembrane domain; Nle, norleucine.

†To whom reprints requests should be addressed at: The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. U17858).

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antisense riboprobe was synthesized by using T7 RNA polymerase. RNase protection assays were performed by hybridizing 25 μg of total RNA to 2–3 × 10^5 cpm of labeled riboprobe at 65°C for 18 h. This was followed by RNase digestion (200 μg of RNase A and 350 units of RNase T1 per ml) at 23°C for 60 min, after which time samples were electrophoresed through 5% polyacrylamide/8 M urea gels.

Radioreceptor Assay. Peptides were synthesized by standard methods (15) and iodinated as reported previously (16). Plasmid DNA corresponding to the full-length CRF-RB in pcDNA1 was prepared by the alkaline-lysis method. COS-M6 cells were transfected with ≈10 μg of this plasmid DNA by the DEAE-dextran method. Two days later, the cells were detached, and crude membrane fractions were prepared and used to measure binding by competitive displacement of ^{125}I-labeled [Nle^{21},Tyr^{32}]-CRF(ovine) (Nle = norleucine) by unlabeled [Nle^{21},Tyr^{32}] CRF(ovine) as described (10). The displacement data were analyzed by using the LIGAND program (17).

cAMP Stimulation in Transfected Cells. One day after transfection of COS-M6 cells with CRF-RB, they were trypsinized, replated in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) into 24- or 48-well tissue culture dishes (Costar), and allowed to recover another 24 h. The medium was changed to DMEM with 0.1% FBS at least 2 h before treatments. The cells were preincubated for 30 min with 0.1 mM 3-isobutyl-1-methylxanthine and then exposed to rat/human CRF or other peptides for 30 min at 37°C. Intracellular cAMP was measured in duplicate from triplicate wells by using an RIA kit (Biomedical Technologies, Stoughton, MA).

Hybridization Histochemistry. Six-week-old male C57BL/6 mice were fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M borate buffer, and a regularly spaced series of 20- to 30-μm frozen sections through brain, heart, duodenum, and testis/epididymis were cut as described (18). Radiolabeled antisense and sense (control) cRNA copies were synthesized from a 1-kb *Bam*HI fragment of CRF-RB cDNA, encompassing 80 bp of the 5′ untranslated region and 926 bp of the CRF-RB coding sequence, that was subcloned into the pBluescript KS vector

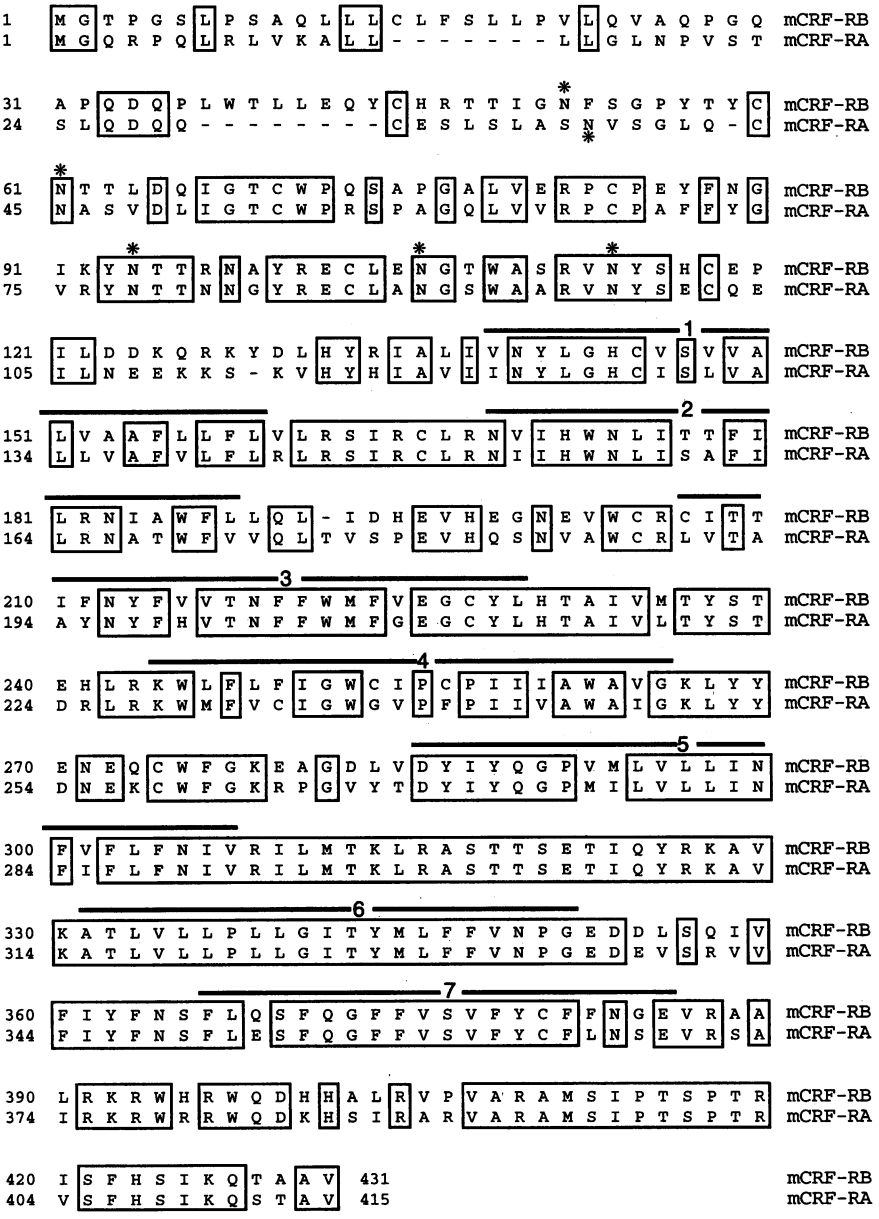

FIG. 1. Amino acid sequence comparison of mouse CRF-RB (mCRF-RB) with mouse CRF-RA (mCRF-RA) (12). The alignment was made by using the Jotun–Hein method with the PAM250 residue-weight table. Putative TMDs are indicated with a solid bar above the sequence. Potential N-glycosylation sites are indicated by an asterisk.

(Stratagene). cRNA probes were labeled by incorporation of [^{35}S]UTP, and *in situ* hybridization was performed as previously described (18). Probes were labeled to specific activities of 1–3 × 10^9 dpm per μg of DNA, and hybridization was carried out under high-stringency conditions (50% formamide with final washes in 0.2× SSC at 70°C).

RESULTS

Cloning of CRF-RB. From a screen of ≈10^6 clones of a mouse genomic library with a CRF-RA probe, nine hybridization-positive phage clones were isolated, and their DNA was prepared for dot-blot hybridization analysis. One of these clones showed a much reduced hybridization signal after a high-stringency wash as compared to that after a low-stringency wash. By Southern blot analysis, the 3.3-kb *Not* I–*Bam*HI fragment located at the 5′ end of this clone retained the hybridization signal even after the high-stringency wash and therefore shared the highest degree of similarity to CRF-RA. This fragment was subcloned and partially sequenced. From the 2.5-kb region, three potential exons were found that were very similar to TMDs-3, -4, and -5 of CRF-RA. These three exons comprised 288 bp, and their sequence was about 75% identical to that of CRF-RA at the nucleotide level and 70% identical at the amino acid level. Thus, this genomic clone was thought to represent a gene closely related to CRF-RA and was designated CRF-RB.

Preliminary RNase protection analysis using a 120-nt probe corresponding to the sequence extending from TMD-3 to intracellular loop 2 indicated that the new receptor was strongly expressed in heart. Therefore, a mouse heart cDNA phage library was screened by hybridization, and one positive clone was identified. This clone, CRF-RB, contained a 2.2-kb insert that included a 1293-bp open reading frame encoding a protein of 431 amino acids.

Receptor Characteristics. Fig. 1 shows the comparison between the amino acid sequences of mouse CRF-RB and mouse CRF-RA. There is a putative signal peptide and five putative N-glycosylation sites in the N-terminal domain in CRF-RB, as well as in CRF-RA. CRF-RB is 16 amino acids longer than CRF-RA (inclusive of putative signal peptides) and, overall, the two receptors are 70% and 68% similar at the nucleotide and amino acid levels, respectively (12). When comparing the sequence of CRF-RB with the sequence of CRF-RA at the amino acid level, there is 79% identity in the seven TMDs and 84% identity in the intracellular loops and the intracellular tail. All but one of the putative intracellular phosphorylation sites in CRF-RA are found in CRF-RB, the missing one being in the C-terminal domain. There is only 60% identity in the extracellular loops and 40% identity in the N-terminal domains. CRF-RB has an extra cysteine in a region of the N terminus, as well as an extra cysteine at the junction of extracellular loop 1 and TMD-3. It is possible that the former cysteine is removed with the putative signal peptide and that the latter cysteine may be included within a TMD, resulting in a total of six cysteines in the extracellular region, as in the other members of this receptor family.

COS-M6 cells transfected with CRF-RB expression vectors produce cell surface proteins that bind CRF with high affinity, as determined by the competitive displacement of bound radioligand. The dissociation constant (K_d) for [Nle^{21},Tyr^{32}]-CRF(ovine) is 1.2 (0.57–2.5) nM (n = 3) (Fig. 2). Growth hormone-releasing factor and vasoactive intestinal peptide do not displace the bound radioligand (data not shown). CRF stimulates the accumulation of intracellular cAMP in transiently transfected COS-M6 cells (Fig. 3) with a half-maximal effective concentration (EC_{50}) of 1.3 ± 0.3 nM (n = 6), and a dose response similar to that of the cloned mouse CRF-RA (data not shown). Suckerfish urotensin I (EC_{50} = 0.7 ± 0.3 nM; n = 3) and sauvagine (EC_{50} = 0.6 ± 0.1 nM; n = 3), which are members of the CRF-peptide family, are highly potent in stimulating intracellular cAMP accumulation, while growth hormone-releasing factor and vasoactive intestinal peptide are ineffective (Fig. 3). Transduction of the CRF signal by the cloned receptor is inhibited by the potent CRF antagonist [DPhe^{12},Nle^{21,38}]hCRF(12–41) (Fig. 3).

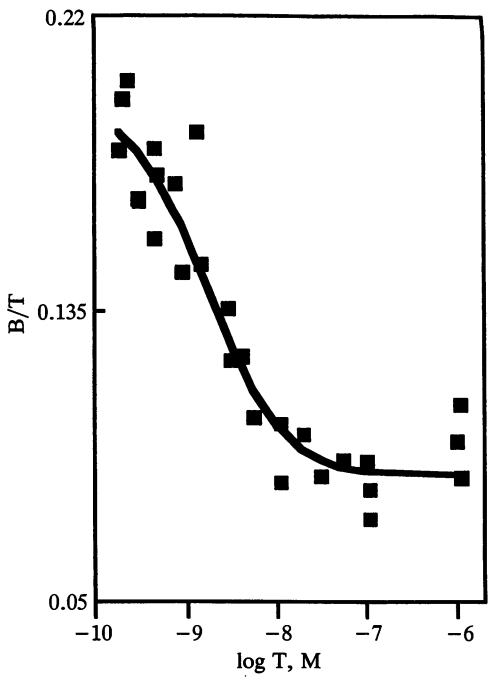

FIG. 2. Competitive displacement by unlabeled [Nle^{21},Tyr^{32}]-CRF(ovine) of ^{125}I-labeled [Nle^{21},Tyr^{32}]CRF(ovine) bound to membranes of COS-M6 cells transfected with CRF-RB cDNA. T, total hormone; B, bound hormone. Data are pooled from three independent experiments.

Tissue Distribution. RNase protection analyses revealed that CRF-RB is expressed in the brain and the heart (Fig. 4). There are multiple protected fragments of CRF-RB in the

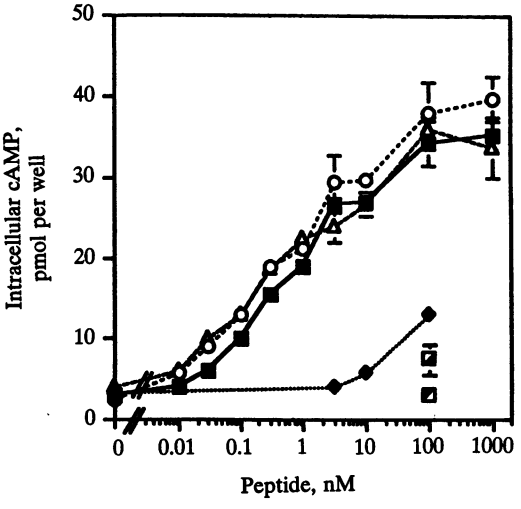

FIG. 3. Accumulation of intracellular cAMP in COS-M6 cells transfected with CRF-RB and stimulated by rat/human CRF (■), sauvagine (○), suckerfish urotensin I (△), human growth hormone-releasing factor [hGRF(1–40)-OH] (◩), and vasoactive intestinal peptide (◪). Data are also shown for the inhibition of the stimulation when the cells are exposed to the antagonist [DPhe^{12},Nle^{21,38}]hCRF(12–41) at 1 μM (◆). Data are from one representative experiment performed as described in *Materials and Methods* and repeated at least twice. The error bars represent the SEM and are smaller than the symbols if not visible.

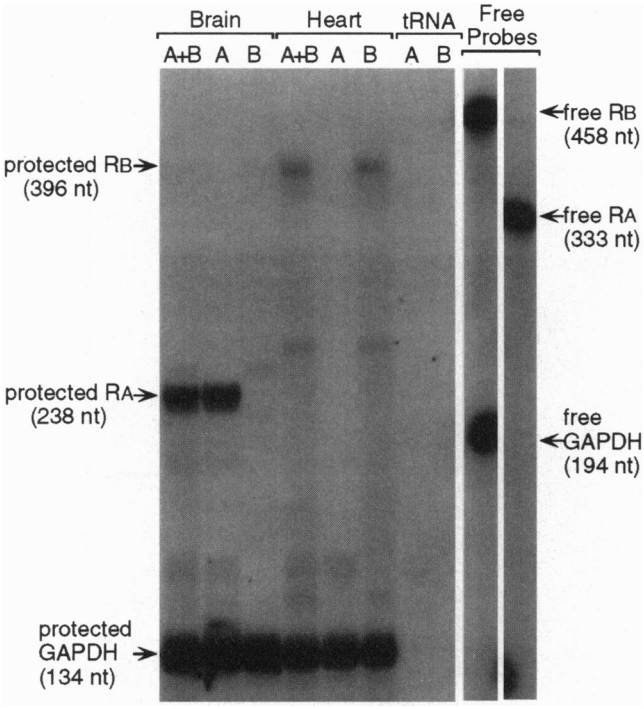

FIG. 4. RNase protection analysis of the expression of CRF-RA and CRF-RB mRNA in mouse brain and heart. The mouse CRF-RA (RA) and CRF-RB (RB) riboprobes (2–3 × 10^5 cpm), individually (lanes indicated as A or B) or together (lanes indicated as A+B), were hybridized with 25 μg of total RNA from mouse whole brain or heart. A riboprobe generated from a mouse glyceraldehyde-3-phosphate dehydrogenase cDNA (GAPDH; 10^4 cpm) was included as an internal loading control, and yeast tRNA was used to monitor nonspecific hybridization.

heart, but the major protected fragment in brain is smaller than that in heart.

The tissue distribution of CRF-RB mRNA was further characterized by *in situ* hybridization histochemistry by using ^{35}S-labeled antisense cRNA probes. Sense-strand cRNAs labeled to similar specific activities failed to show any suggestion of positive localizations when applied to tissue sections adjoining those in which antisense probes revealed robust hybridization signals. Consistent with cloning and protection assay data, CRF-RB transcripts were detected in heart, where labeling appeared most prominently over perivascular cells (Fig. 5), as well as in the epicardium (data not shown). In the male reproductive tract, CRF-RB mRNA was localized principally in stromal tissue of the epididymis, while labeling in testis was at or near background levels. The CRF-RB mRNA signal over duodenum appeared as a dense band of silver grains over the submucosal layer and additionally, over isolated nonepithelial cells at the base of the villi. In brain, CRF-RB mRNA displayed a rather restricted distribution, which contrasted in extent and topography with that for CRF-RA mRNA (14). In the septal region, for example, CRF-RB mRNA was expressed in circumscribed aspects of the lateral septal nucleus (Fig. 5), while the CRF-RA transcript is seen over the medial septal complex. Other major sites of CRF-RB mRNA expression in the forebrain included circumscribed aspects of the olfactory bulb, preoptic region, hypothalamus, and amygdala. In each of these areas, the pattern of CRF-RB expression was distinct from that of CRF-RA (14). It appears unlikely that major species differences in CRF-R distribution are at play, since the mouse CRF-RB probe employed here yielded similar patterns of hybridization in mouse and rat brain.

DISCUSSION

Using a probe derived from our original cloned CRF-R, CRF-RA, we have isolated a second CRF-R encoded by a distinct gene. Although longer than CRF-RA and 68% similar at the amino acid level, most fundamental structural characteristics have been conserved in CRF-RB. The main difference between the two receptors is found in the N-terminal domain in which there are 16 extra amino acids in CRF-RB compared with CRF-RA and significant, nonconservative amino acid changes. It is interesting to note that, on the basis of the genomic sequence of the mouse CRF-RA[¶] the divergence of the amino acid sequences of the two receptors occurs close to

[¶]Chen, R., Lewis, K. A., Perrin, M. H., & Vale, W. W., Proceedings of the 76th Annual Meeting of the Endocrine Society, Anaheim, CA, June 15–18, 1994, p. 217 (abstr. 67).

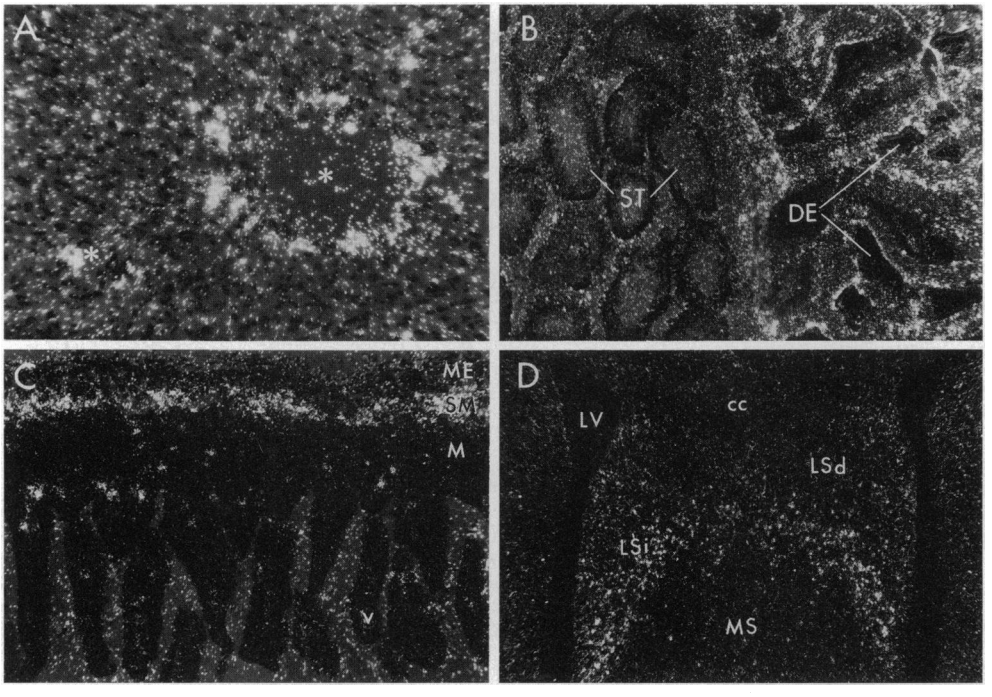

FIG. 5. *In situ* hybridization histochemical localization of CRF-RB in mouse tissues. Dark-field photomicrographs showing specific hybridization signal over arterioles (asterisks) in the myocardium (*A*); stromal but not ductal (DE) aspects of epididymis (right); note the lack of robust labeling of testis (left) (*B*); duodenum, where labeling appears as a dense band over the submucosal (SM) layer, and over isolated nonepithelial cells of the mucosa (M) (*C*); and brain, specifically the intermediate part of the lateral septal nucleus (LSi) (*D*). Other abbreviations: ST, seminiferous tubules; ME, external muscle layer; v, villus; LSd, lateral septal nucleus, dorsal part; MS, medial septal nucleus; cc, corpus callosum; LV, lateral ventricle. (Magnifications: *A*, ×90; *B*, ×110; *C*, ×55; *D*, ×20.)

the second intron/exon junction near the N-terminus of CRF-RA, raising the possibility that some of the sequence differences between the two receptors could result from alternative exon utilization in CRF-RB. Indeed, the presence of multiple protected RNA species in the heart and the smaller size of the major protected band in the brain when using N-terminal riboprobes for CRF-RB in the RNase protection assay (Fig. 4) is consistent with the existence of splice variants of this receptor.

A major determinant of the coupling of 7-TMD receptors to the GTP-binding proteins and subsequently, to adenylate cyclase is thought to reside in the third intracellular loop (20), although in some receptors there appears to be a contribution from other components as well (21–23). Because of the similarities between the third intracellular loops and the C termini of the two receptors, their coupling and signal transduction properties are predicted to be very similar. Because the N-termini of the two receptors differ and since a major binding determinant may reside in the N-terminal domain, as has been proposed for the PTH receptor (24), it was possible that the binding specificities might differ. The results from the cAMP dose response data demonstrate that sauvagine and urotensin are effective, in the nanomolar range, at stimulating cAMP accumulation and that they appear somewhat more potent than CRF. By contrast, sauvagine has been reported to be slightly less potent than CRF in stimulating cAMP in cells transfected with CRF-RA (13).

CRF exerts powerful effects on peripheral organs by modulating the autonomic nervous system and also plays a role within many tissues as a local mediator. As revealed by RNase protection analyses, CRF-RB is abundantly expressed in the heart, which has been reported also to express CRF mRNA (25). In the isolated, perfused heart, the addition of CRF into the left atrium induces a prolonged dilatory effect on coronary arteries, transiently produces a positive ionotropic effect, and stimulates the secretion of atrial natriuretic peptide (26, 27). The finding of CRF-RB expression in the blood vessels of the heart raises the possibility that CRF or other natural or pharmacologic ligands for this receptor might regulate cardiac perfusion. Furthermore, it will be interesting to examine other vascular beds, such as that of the superior mesenteric artery, which is known to be dilated by CRF and related ligands.

The CRF-RB gene is expressed in the gastrointestinal tract as shown by the presence of CRF-RB mRNA in the submucosal and deeper regions of the duodenum. This receptor may mediate some of the direct stimulatory effects of CRF on the gastrointestinal tract that have been described; for example, CRF acts on the gut *in vitro* to depolarize myenteric neurons in the small intestine (28). Results from *in vivo* studies with intravenously administered CRF and CRF antagonists are consistent with a direct effect by CRF to control gastric emptying and intestinal motility (29–31). Accordingly, CRF immunostaining is present at many levels of the gastrointestinal tract (32–34). The presence of CRF-RB in the epididymis may enable local communication with spermatozoa, which are reported to possess immunoreactive CRF (35).

CRF-RB is present in the brain; however, the major protected species appears to differ from the transcript that we cloned from heart and may have a different N-terminal domain. *In situ* hybridization studies indicate that mRNA related to CRF-RB has a restricted distribution in the central nervous system that differs considerably from that of CRF-RA. Thus, the receptors derived from the two genes, A and B, with their distinct tissue distributions and structural diversity, especially in the extracellular domains, are likely to subserve disparate biological roles.

Note Added in Proof. While this manuscript was in press, Lovenberg *et al.* (19) reported the sequence of a clone (obtained by PCR) of the rat homolog of CRF-RB and the cloning of a rat hypothalamic cDNA corresponding to a splice variant of CRF-RB encoding a shorter receptor protein of 411 amino acids. When expressed in Ltk^- cells, the shorter receptor exhibited no detectable binding to CRF and showed an EC_{50} of 20 nM, 0.5 nM, and 2 nM for CRF-, sauvagine-, and urotensin-stimulated intracellular cAMP accumulation, respectively.

M.P., C.D., and R.C. should be considered cofirst authors by virtue of their unique contributions to this work. We thank R. Kaiser and J. Rivier for the synthetic peptides; J. Morehead, M. Burns, J. Yehling, and C. Arias for excellent technical assistance; K. Lewis and Dr. K.-F. Lee for helpful discussions; and S. Guerra, B. Coyne, D. Johns, and D. Dalton for manuscript preparation. We also thank Dr. L. Chen, Baylor College of Medicine, for the amplified mouse heart library. This research was supported in part by National Institutes of Health Program Project Grant DK26741, the Foundation for Research, the Adler Foundation, and the Mellon Foundation.

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