General and Comparative Endocrinology 116, 461–477 (1999)
Article ID gcen.1999.7386, available online at http://www.idealibrary.com on IDEAL®

Differential Expression of Corticotropin-Releasing Factor (CRF) and Urotensin I Precursor Genes, and Evidence of CRF Gene Expression Regulated by Cortisol in Goldfish Brain

Nicholas J. Bernier, Xinwei Lin, and Richard E. Peter

Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

Accepted September 16, 1999

Corticotropin-releasing factor (CRF) and urotensin I (UI) precursor cDNAs were cloned and sequenced from a goldfish brain cDNA library in order to investigate the distribution of CRF and UI mRNAs in goldfish brain and the regulation of CRF and UI gene expression. The CRF (966-bp) and UI (769-bp) cDNAs encode 163- and 146-amino acid precursors, respectively, and consist of a signal peptide sequence, a cryptic region, and a 41-amino acid mature peptide at the carboxy terminal. The deduced amino acid sequences of the CRF and UI peptides exhibit a sequence identity of 54%. Northern blot analysis revealed a single size of CRF (1.3 kb) and UI (2.0 kb) mRNAs, which are expressed in the telencephalon–preoptic, hypothalamic, optic tectum–thalamus, and posterior brain regions, but not in the pituitary. In addition, while the CRF gene is strongly expressed in the olfactory bulbs, the UI gene is not. In brain regions in which both genes are expressed, the mRNA levels of CRF were three- to sevenfold higher that those of UI. While the low expression levels of the UI gene prevented further analysis of its regulation, the regulation of CRF gene expression by cortisol was examined. In response to intraperitoneal implants of cortisol (300 μg/g BW) the level of CRF mRNA in the telencephalon–preoptic region decreased to 69% of control values at 6 and 24 h posttreatment. In sham-treated fish, in parallel with a transient injection stress-elicited increase in plasma cortisol, CRF mRNA levels declined to 72% of control value at 6 h postinjection and recovered after 24 h. Injection of the glucocorticoid antagonist, RU-486 (100 μg/g BW), prevented the reduction in CRF gene expression associated with the injection stress at 6 h and increased CRF mRNA levels to 145% of control value after 24 h. In contrast, the various implants had no effect on CRF mRNA levels in either the hypothalamus or the optic tectum–thalamus region. These results provide evidence of differential expression of the CRF and UI genes in hypothalamic and extrahypothalamic regions of goldfish brain. Furthermore, they demonstrate that stress levels of plasma cortisol can lead to a decrease in CRF gene expression that is mediated by glucocorticoid receptors in the telencephalon–preoptic region and give an indication of the regional specificity of the regulation of CRF gene expression by cortisol.

Key Words: corticotropin-releasing factor precursor; urotensin I precursor; cDNA; gene expression; mRNA distribution; cortisol; negative feedback regulation; brain; goldfish.

Corticotropin-releasing factor (CRF) and urotensin I (UI) are homologous neuropeptides originally isolated from sheep hypothalami (Vale *et al.*, 1981) and fish urophysis (Ichikawa *et al.*, 1982; Lederis *et al.*, 1982), respectively. Together with amphibian sauvagine (Montecucchi and Henschen, 1981) and mammalian urocortin (Vaughan *et al.*, 1995), CRF and UI are members of a peptide family that share partial sequence identity,

0016-6480/99 $30.00

pharmacological properties, and physiological functions (for review see Lovejoy and Balment, 1999). Primary among the physiological actions of CRF-related peptides in fish and mammals is their stimulatory effect on the release of adrenocorticotropic hormone (ACTH) from the pituitary (Fryer *et al.,* 1983; Vaughan *et al.,* 1995). In fact, CRF is now generally acknowledged to be the principle regulator of the hypothalamic–pituitary–adrenal (HPA) axis in mammals and to play a major role in the integration of the body's response to stress (Turnbull and Rivier, 1997). In fish, although CRF plays an important role in the control of the hypothalamic–pituitary–interrenal (HPI) axis (for review see Lederis *et al.,* 1994), its dominance among a diverse array of corticotropin-releasing peptides in the neuroendocrine integration of the stress response remains to be demonstrated (Fryer, 1989; Wendelaar Bonga, 1997).

Following the observation that UI is more potent than CRF in the release of ACTH from fish pituitaries and that the hypothalamus is an extraurophysial site of UI synthesis (Fryer *et al.,* 1983; Woo *et al.,* 1985), it was suggested that UI may act as CRF in teleost fish (Lederis *et al.,* 1985). However, several studies have provided evidence that CRF- and UI-like immunoreactivity occurs in separate neuronal populations in the teleost brain (Yulis *et al.,* 1986; McMaster and Lederis, 1988; Fryer, 1989). Moreover, CRF precursor cDNAs have been isolated from hypothalamic cDNA libraries of white sucker (*Catostomus commersoni;* Okawara *et al.,* 1988; Morley *et al.,* 1991) and sockeye salmon (*Oncorhynchus nerka;* Ando *et al.,* 1999).

Hypothalamic lesioning studies in goldfish (*Carassius auratus*) first identified the nucleus preopticus (NPO) and the nucleus lateral tuberis (NLT) as sources of hypothalamic factors involved in the control of ACTH (Fryer and Peter, 1977a). Subsequent investigations showed that the NPO and the NLT are the principle sites of CRF- and UI-like peptides in teleosts (Yulis *et al.,* 1986; McMaster and Lederis, 1988; Olivereau and Olivereau, 1988; Fryer, 1989; Lederis *et al.,* 1994). In addition, these studies revealed a complex network of neuronal fibers immunoreactive to UI and CRF scattered throughout the central nervous system (Lederis *et al.,* 1985, 1994; Yulis *et al.,* 1986). Northern blot and *in situ* hybridization experiments have identified the NPO and the NLT as locations of CRF and UI synthesis in the brain of teleosts (Morley *et al.,* 1991; Okawara *et al.,* 1992; Ando *et al.,* 1999). So far, however, the potential synthesis of CRF or UI outside of these two hypothalamic nuclei has not been investigated.

It is well established that CRF gene expression in the paraventricular nucleus (PVN), the mammalian equivalent to the NPO, is negatively regulated by glucocorticoids (Jingami *et al.,* 1985; Imaki *et al.,* 1991). Similarly, several lines of evidence suggest that cortisol may have a regulatory impact on CRF synthesis in fish (Fryer and Peter, 1977b; Fryer and Boudreault-Châteauvert, 1981; Olivereau and Olivereau, 1990; Morley *et al.,* 1991). However, the direct effects of elevated circulating cortisol levels or of glucocorticoid receptor blockade on CRF gene expression in fish are not known. Similarly, although pharmacological adrenalectomy results in an increase in UI immunoreactivity in the NLT of goldfish (Fryer, 1989), the effects of cortisol on UI mRNA levels have not been ascertained.

In the present study, to explore further the gene expression pattern of CRF-related peptides in fish, cDNAs for CRF and UI were cloned from a goldfish brain cDNA library. Once oligonucleotide probes were developed, we determined the general distribution of the mRNAs for CRF and UI in discrete brain regions of goldfish. In addition, the potential impact of glucocorticoids on CRF and UI mRNA levels in hypothalamic and extrahypothalamic brain regions was investigated in goldfish given intraperitoneal implants of either cortisol or RU-486. RU-486 is a potent glucocorticoid antagonist in mammals (Gaillard *et al.,* 1984; Healy *et al.,* 1985), which has previously been shown to bind with high affinity to cortisol receptors in fish cells (Lee and Bols, 1989). Therefore, RU-486 may prove a useful tool for providing evidence of negative feedback regulation by cortisol of CRF or UI synthesis.

MATERIALS AND METHODS

Animals

Mixed-sex goldfish (*C. auratus*) of the common or comet variety weighing between 12 and 38 g (22.2 $\pm$ 0.4 g, mean $\pm$ SEM, experimental $n = 256$) were obtained from Mount Parnell Fisheries (Mercersburg, PA) and maintained in 800-liter flow-through fiberglass tanks

at 17°C under a simulated natural photoperiod at Edmonton, AB, Canada. The fish were fed *ad libitum* once daily with commercially prepared Unifeed Nu-Way trout ration (United Grain Growers, Okotoks, AB, Canada).

Preparation of Total RNA

Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222; Syndel, Vancouver, BC, Canada) prior to decapitation and dissection of the tissues. Total RNA was extracted from goldfish brain and pituitary using Trizol Reagent (Life Technologies, Gaithersburg, MD) based on the acid guanidinium thiocyanate–phenol–chloroform extraction method. RNA concentrations were determined by optical density at 260 nm, and ethidium bromide staining of 18s and 28s ribosomal bands on a denaturing agarose gel verified integrity of the RNA.

Cloning of CRF and UI cDNAs

For cloning of goldfish CRF and UI cDNAs, reverse transcription-polymerase chain reaction (RT-PCR) was used to prepare DNA probes for screening a goldfish brain cDNA library. Briefly, total RNA was extracted from goldfish forebrain (telencephalon and hypothalamus) as above and 5 μg total RNA was reverse transcribed to cDNA using the SuperScript Preamplification System (Life Technologies). The oligonucleotide primers used for PCR were synthesized by Life Technologies and the sequences are shown in Table 1. For CRF, forward primer P1 and reverse primer P2 were designed on the basis of highly conserved coding sequences of CRF precursors in mammals and white sucker (Okawara *et al.*, 1988). For UI, forward primer P5 and reverse primer P6 were designed on the basis of partial nucleotide sequences encoding goldfish UI peptide (Vaughan *et al.*, 1995). With either set of primers, 35 cycles of PCR amplification were performed with denaturation for 1 min at 95°C, annealing for 1 min at 53°C, extension for 1 min at 73°C, and final extension for 10 min at 73°C after the last cycle. The PCR products were separated by agarose gel, and the bands of desired size were excised and purified using a Geneclean II kit (Bio 101, La Jolla, CA). The purified cDNA fragments, P1/P2 (317 bp) and P5/P6 (129 bp) PCR products (Fig. 1), were subcloned into pGEM-T vector (Promega, Madison, WI). Recombinant plasmid DNA containing the cDNA inserts were purified by an alkaline lysis method (Birnboim, 1983) and the isolated plasmid DNAs were sequenced on an Applied Biosystems Automated Sequencer (Perkin–Elmer, Norwalk, CT). Both strands of cloned DNA were sequenced in opposite directions using T7 and M13 sequencing primers that flank the inserted cDNA. The nucleotide sequence analyses indicated that the cloned P1/P2 and P5/P6 PCR products contained partial coding regions for precursors with CRF and UI sequences at their C-terminus, respectively. These DNA fragments were then used as probes to screen the cDNA library.

A goldfish brain cDNA library (kindly provided by Dr. H. R. Habibi, University of Calcary, Calcary, AB, Canada) was constructed using the ZAP-cDNA synthesis and Gigapack II Gold packaging extract kits (Stratagene, La Jolla, CA). The library was amplified once to a titer of 6×10^9 plaque-forming units/ml before being transferred to Hybond-N discs (Amersham Life Science, Buckinghamshire, England) at a density of 2×10^4 plaques/filter. The filters were then probed with the P1/P2 (CRF) or P5/P6 (UI) PCR products (Fig. 1) prepared as described above. The DNA probes were labeled with [α-^{32}P]dCTP using a T7QuickPrime kit (Pharmacia Biotech, Baie d'Urfé, QC, Canada). Hybridization of the filters was performed using the methods of Church and Gilbert (1984). In brief, the filters were prehybridized in a hybridization solution (0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA) for at least 1 h at 65°C. The hybridization solution was then changed and the labeled probe was added. After hybridization for at least 16 h at 65°C, the filters were washed four times with a washing solution (40 mM

TABLE 1
Primers Used for Amplification of Corticotropin-Releasing Factor and Urotensin I cDNA Fragments from the Brain of Goldfish

Primers	Sequences
P1	5′TAGGGGAGGAGTACTTCATCCG3′
P2	5′GCGGTTGCTGTGAGCTTGCTG3′
P3	5′ACGCACAGATTCTCCTCGCCAC3′
P4	5′CAGAATATTCATGTCTGTGCTAAATG3′
P5	5′AACGACGATCCTCCGATCTCC3′
P6	5′CTTCCCAACCTCATCTAG3′
P7	5′AAGCGCGCAGCTGTGTCCAGC3′

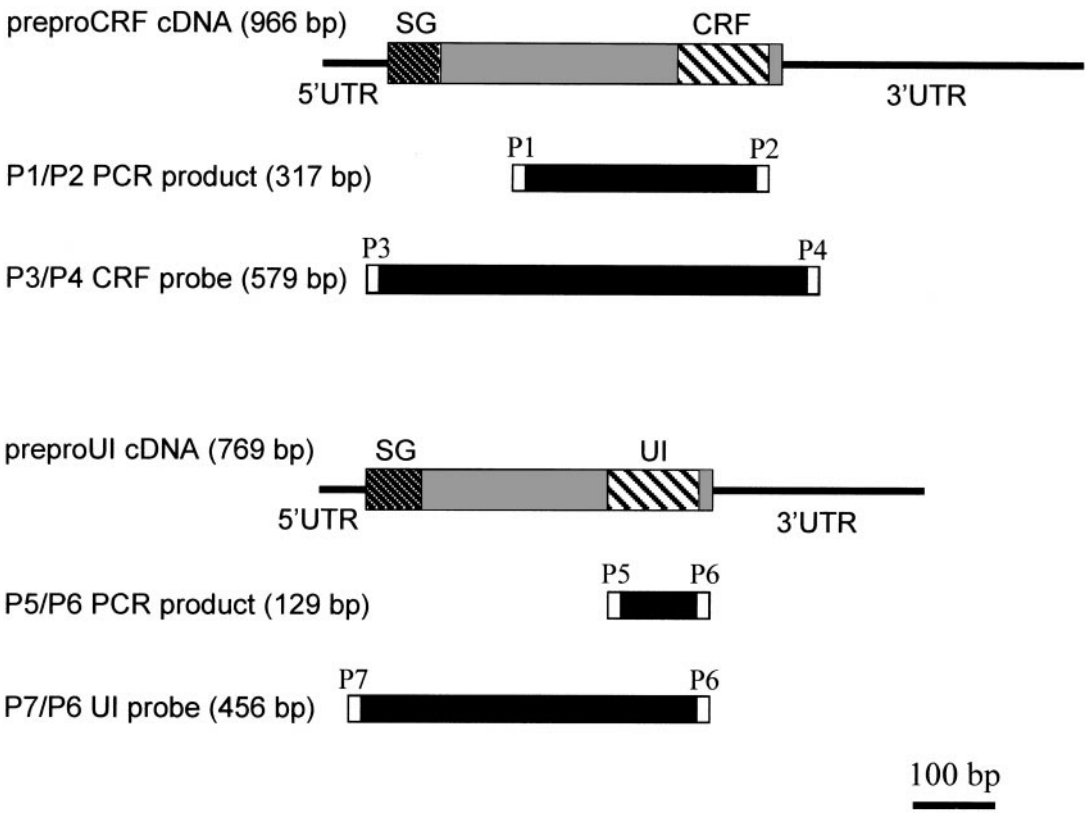

FIG. 1. Schematic structure of goldfish preprocorticotropin-releasing factor and preprourotensin I cDNAs and position of primers used for PCR assay. PCR amplification of cDNA with primer set P1 and P2 resulted in a 317-bp product, and amplification of cDNA with primer set P5 and P6 resulted in a 129-bp product. P1/P2 and P5/P6 PCR products were used as probes to screen a goldfish brain cDNA library. PCR amplification of cDNA with primer set P3 and P4 resulted in a 579-bp product, and amplification of cDNA with primer set P7 and P6 resulted in a 456-bp product. P3/P4 and P7/P6 PCR products were used as probes for Northern blot analysis of CRF and UI mRNA levels, respectively. Abbreviations: CRF, corticotropin-releasing factor; UI, urotensin I; 5′UTR, 5′-untranslated region; 3′UTR, 3′-untranslated region; SG, signal peptide; P1–P7, gene-specific primers (see Table 1 for sequences).

$NaHPO_4$, pH 7.2, 1 mM EDTA, and 1% SDS) and autoradiographed on Kodak X-OMAT X-ray film with intensifying screens at −80°C. With each probe, a total of 2×10^5 clones were screened out of which 3 positive CRF and 5 positive UI clones were isolated and subjected to secondary screening. Two positive CRF and UI clones were obtained from the secondary screening. These clones were amplified and subjected to *in vivo* excision using the helper phage rescue method according to the instructions of the ZAP-cDNA synthesis kit (Stratagene). The plasmid DNA containing the cDNA inserts of desired size were purified and sequenced as above. Sequencing was carried out on both strands using the T7 and T3 sequencing primers flanking the inserted cDNA. In addition, in order to obtain complete overlapping sequences, the preproCRF cDNA was sequenced with the gene-specific primers P1 and P2, and the preproUI cDNA was sequenced with the gene-specific primers P5 and P6.

Expression Pattern of CRF and UI Genes in Goldfish Brain

Northern blot analysis was carried out for detection of CRF and UI gene expression in discrete brain areas of goldfish. Tissues of five brain areas, olfactory bulbs, telencephalon–preoptic region, hypothalamus, optic tectum–thalamus region, and posterior brain (including cerebellum, medulla, and spinal cord), and pituitary were freshly excised and homogenized for extraction of total RNA using Trizol Reagent as above.

Twenty micrograms of total RNA from each individual tissue were denatured at 65°C for 10 min in 0.2 M Mops (pH 7.0), containing 2.2 M formamide, 8% formaldehyde (37%), 50 mM sodium acetate, and 10 mM EDTA, and fractionated by electrophoresis in a denaturing 1.5% agarose gel. The gel was blotted onto Hybond-N membrane by capillary transfer and the RNA was fixed by baking at 80°C for 1 h and crosslinked by UV irradiation for 30 s. For hybridization of the membranes, probes were synthesized by PCR using plasmid DNA as templates with the gene-specific primers P3 and P4 for preproCRF cDNA (P3/P4 CRF probe) or P7 and P6 for preproUI cDNA (P7/P6 UI probe; Fig. 1). The probes were then purified by agarose gel electrophoresis and Geneclean II kit. Probe labeling and hybridization of the membranes were carried out as above. The specific activity of the CRF and UI probes used for Northern blot analysis was consistently about 1.6×10^8 dpm/μg. Signal detection was achieved by exposing the membranes to a PhosphorImager screen for 2 days (Molecular Dynamics, Sunnyvale, CA) and quantified by ImageQuant software (Molecular Dynamics). To serve as an internal control, the membranes were stripped and reprobed with a [α-^{32}P]dCTP-labeled partial cDNA for goldfish β-actin (Peyon *et al.*, 1998). The mRNA levels for each individual tissue were expressed as a ratio between CRF mRNA and β-actin mRNA and between UI mRNA and β-actin mRNA.

Effects of Cortisol and RU-486 on CRF and UI Gene Expression

Nine groups of 24 fish each were adapted for a 2-week period to individual 80-liter fiberglass tanks. After this acclimation period, two groups of fish each received either a blank (sham treatment), cortisol (hydrocortisone; Sigma Chemical, St. Louis, MO), or RU-486 (mifepristone; Sigma) silastic pellet, and three control groups were left undisturbed. The solid silastic pellets were manufactured as previously described (Pankhurst *et al.*, 1986) and implanted intraperitoneally through a 2- to 3-mm incision in the body wall under MS-222 (0.02%) anesthesia between 9:30 and 11:00 AM. Fish implanted with the cortisol pellets received a dose of 300 μg/g body weight (BW) and those treated with RU-486 received a dose of 100 μg/g BW. Six and 24 h after receiving the implants, fish were terminally anesthetized (0.05% MS-222) for collection of blood and brain tissues. One control group was sampled at 9:00 AM (time 0 control), immediately prior to the implantation of the experimental groups, and the two other control groups were sampled 6 and 24 h later. Blood was collected by caudal puncture using 25-gauge needles and centrifuged at 10,000*g* for 5 min, and the separated plasma was stored at −20°C for later analysis of cortisol. Brains were obtained by decapitation and regionally dissected to determine the expression levels of CRF and UI mRNAs.

Concentrations of plasma cortisol, the primary circulating corticosteroid in goldfish, were determined by radioimmunoassay (RIA) using a commercially available kit (ImmuChem Coated Tube Cortisol ^{125}I RIA Kit; ICN Biochemicals, Costa Mesa, CA). The validity of the RIA for measuring cortisol titers in goldfish plasma was assessed with the determination of displacement curves obtained with serial dilutions of plasma samples. The cross-reaction of RU-486 with the cortisol antibody was assessed to check the specificity of the assay. The intraassay coefficient of variation was 3.3% ($n = 8$).

CRF and UI mRNA levels were assessed by Northern blot analysis in the telencephalon–preoptic region, the hypothalamus, and the optic tectum–thalamus region. In addition, CRF mRNA levels were examined in the olfactory bulbs. In order to obtain at least 30 μg total RNA from each brain region, the telencephalon–preoptic, the hypothalamus, and the optic tectum–thalamus tissues were pooled two by two and the olfactory bulbs three by three prior to RNA extraction. Total RNAs, 10 and 20 μg for the CRF and UI gels, respectively, were denatured and subjected to electrophoresis as above. After electrophoresis, RNAs were transferred to Hybond-N membranes, fixed, and UV crosslinked. The RNA blots were then hybridized with either the P3/P4 CRF probe or the P7/P6 UI probe. Signal detection was achieved by exposing the CRF and UI membranes to a PhosphorImager screen for 1 and 3 days, respectively, and quantified by ImageQuant program. The CRF and UI mRNA levels were expressed as a ratio to the hybridization signal for β-actin mRNA (internal control) and then normalized as a percentage of the time 0 control group values for each individual tissue analyzed.

Statistics

Results are presented as mean values + one standard error of the mean (SE) throughout. Among the three control groups or between the four different treatments at either the 6- or 24-h sampling time, statistical differences were determined using a one-way analysis of variance followed by a Tukey test for multiple comparisons. The significance level for all statistical tests was $P < 0.05$.

RESULTS

Cloning of CRF and UI cDNAs

Screening of a goldfish brain cDNA library with a probe for the coding region of a partial preproCRF cDNA (P1/P2 PCR product; Fig. 1) identified three positive clones. After secondary screening, two clones were characterized by nucleotide sequence analysis. Both clones contained the identical nucleotide sequence. Sequence analysis confirmed the identity of the clones as a preproCRF cDNA since the deduced protein sequence contains CRF at the C-terminus. The 966-nucleotide sequence of goldfish preproCRF cDNA (GenBank Accession No. AF098629) and the deduced amino acid sequence are shown in Fig. 2A. The cDNA is comprised of a 90-bp 5′-untranslated region, 489 bases of open reading frame, and a 387-bp 3′-untranslated region, which contains a polyadenylation signal (AATAAA) and a poly(A) tail. The open reading frame encodes the 163-amino acid goldfish preproCRF, which consists of a putative signal peptide, a cryptic region, and the carboxyl terminus 41-amino acid sequence of CRF. The potential 24-amino acid signal peptide at the amino terminus was determined by the (−3, −1)-rule for predicting the cleavage site between a signal sequence and the mature exported protein (von Heijne, 1986). Goldfish CRF is flanked by the potential proteolytic processing and cleavage signals Arg-Xaa-Arg-Arg at the N-terminus and by Gly-Lys at the C-terminus. The presence of Gly adjacent to the C-terminus is indicative of carboxy-terminal amidation.

Screening of the goldfish brain cDNA library using a partial preproUI cDNA (P5/P6 PCR product; Fig. 1) as probe identified five positive clones. After secondary screening, two clones were characterized by nucleotide sequence analysis. Both clones were indistinguishable. The identity of the sequence indicated a preproUI cDNA since the deduced protein sequence contains UI at its C-terminus. The 769-nucleotide sequence of goldfish preproUI cDNA (GenBank Accession No. AF129115) and the deduced amino acid sequence are shown in Fig. 2B. The cDNA comprises 58 bp of 5′-untranslated region, 438 bases of open reading frame, and a 273-bp 3′-untranslated region, which contains both a common (AATAAA) and modified (ATTAAA) polyadenylation signal and a poly(A) tail. The deduced 146-amino acid goldfish UI precursor consists of a putative signal peptide sequence of 24 amino acids at its amino terminus (von Heijne, 1986), a cryptic region, and the carboxyl terminus 41-amino acid sequence of UI. The double-basic residues Lys-Arg adjacent to the N-terminus of UI indicate a potential enzymatic cleavage site. The Gly-Lys residues following the C-terminus of UI are indicative of a proteolytic processing site and of C-terminal amidation.

The deduced amino acid sequence of the goldfish CRF precursor is closely related to that of the white sucker CRF_1 precursor (Table 2). Goldfish CRF and white sucker CRF_1 peptides differ by a single Met-Leu substitution at amino acid position 27. The predicted goldfish UI peptide has an amino acid sequence identical to the one determined in common carp, *Cyprinus carpio* (Ichikawa *et al.*, 1982), and the goldfish UI precursor also shares its highest degree of sequence identity with the UI precursor from the same species. Overall, a multiple amino acid sequence alignment between goldfish and other vertebrate CRF-related precursors and peptides revealed that goldfish preproCRF and its mature peptide exhibit higher identities with CRF sequences than with other CRF-related peptides. Similarly, goldfish UI precursor and peptide show a higher degree of identity with other UI sequences than with the remaining members of the CRF peptide family.

Expression Pattern of CRF and UI Genes in Goldfish Brain

Northern blot analysis of total RNA using the P3/P4 CRF probe (Fig. 1) identified a single gene transcript of approximately 1.3 kb for the CRF precursor in discrete

A

```
-90                                     atcaactggaagacagacgctcctcgcaaagttcaaaaaccatccaaggatataccaattacgcacagattctcctcgccactttttgac  -1
  1   ATG AAG CTC AAT TTT CTC GTC ACC ACC GTG GCT CTG CTC GTT GCC TTT CCA CCA CCG TAT   60
  1    M   K   L   N   F   L   V   T   T   V   A   L   L   V   A   F   P   P   P   Y    20
 61   GAA TGT AGA GCC ATC GAA GGC AGC TCC AAC CAG CCA GCC ACG GAC CCC GAT GGA GAG CGA  120
 21    E   C   R   A   I   E   G   S   S   N   Q   P   A   T   D   P   D   G   E   R    40
121   CAG TCC CCG CCG GTT TTG GCA CGC TTA GGG GAG GAG TAC TTC ATC CGG CTC GGT AAC AGA  180
 41    Q   S   P   P   V   L   A   R   L   G   E   E   Y   F   I   R   L   G   N   R    60
181   AAC CAG AAT TAT CTC CGA TCC CCA GCC GAC AGC TTC CCC GAG ACA TCC CAG TAT TCC AAA  240
 61    N   Q   N   Y   L   R   S   P   A   D   S   F   P   E   T   S   Q   Y   S   K    80
241   AGA GCA CTG CAG CTC CAG TTA ACG CAG CGT CTG TTG GAG GGG AAA GTT GGG AAC ATC GGT  300
 81    R   A   L   Q   L   Q   L   T   Q   R   L   L   E   G   K   V   G   N   I   G   100
301   CGC TTG GAT GGC AAT TAC GCG CTC CGG GCG CTC GAC TCA GTG GAG AGG GAG CGC AGG TCG  360
101    R   L   D   G   N   Y   A   L   R   A   L   D   S   V   E   R   E   R   R   S   120
361   GAG GAG CCG CCG ATT TCC CTG GAT CTG ACC TTT CAT CTG CTA CGA GAA GTA CTG GAG ATG  420
121    E   E   P   P   I   S   L   D   L   T   F   H   L   L   R   E   V   L   E   M   140
421   GCC AGA GCC GAA CAA ATG GCC CAG CAA GCT CAC AGC AAC CGC AAA ATG ATG GAA ATA TTC  480
141    A   R   A   E   Q   M   A   Q   Q   A   H   S   N   R   K   M   M   E   I   F   160
481   GGG AAG TAA ccatgagcaaacccattagccaaagatatttttacatttagcacagacatgaatattctgtaccatagtgctgcttttccatcatgatctatttataccggtg  592
161    G   K   *                                                                          163
593   acttatttatttgtagatatttcactgaagacgaatagagcacactaggtgtatatgaagcgcaaaactgtcatcactctccatcaatgttattgtataagtgatcattcagaactgtag  712
713   atgaagaaaacgtttgagtttctttaaaaaaaaagacaatattttccagtactgtattttgcttaatttatctgacagacttgagtttcattgtaacatacagtacattgtttaagtttgttA  835
836   ATAAActaatgagcaaccattcaaaaaaaaaaaaaaaaaa                                        876
```

B

```
-58                                                     cttacaagtacttttgaagcaacagaagacgaccgagaagcgcgcagctgtgtccagc  -1
  1   ATG AAG CCC GTC CCT TTG GTC CTG CTC ATA ACT TCA GTC TTA CTG ACC ACC CAC ATC CCG   60
  1    M   K   P   V   P   L   V   L   L   I   T   S   V   L   L   T   T   H   I   P    20
 61   CTG AGC ACC TGT CGA CCC CGC GAC CTG AGC CTC GTG AAC AGC CAG CTA GAC GAC GTG CTG  120
 21    L   S   T   C   R   P   R   D   L   S   L   V   N   S   Q   L   D   D   V   L    40
121   TCA AAC GGG GCA GGA GAC GAC GCT ATG TCC TAC CTC GTG GGT GAA AAA CTC CTT CAG TAT  180
 41    S   N   G   A   G   D   D   A   M   S   Y   L   V   G   E   K   L   L   Q   Y    60
181   TTG CAA AGA AAC CTC GGA GCG CAG AAG GCA AGC GGT GTC CTG CAT CTC CCG CAC TTC CCC  240
 61    L   Q   R   N   L   G   A   Q   K   A   S   G   V   L   H   L   P   H   F   P    80
241   GCG GCG CAG CTC CGC TCT CCT CAC GAG GAC AGC AGC TTA GAA GAG CTC ACG GAG TTT TCC  300
 81    A   A   Q   L   R   S   P   H   E   D   S   S   L   E   E   L   T   E   F   S   100
301   AAA CGG AAC GAC GAT CCT CCG ATC TCC ATC GAC CTC ACC TTC CAC CTG CTG AGA AAC ATG  360
101    K   R   N   D   D   P   P   I   S   I   D   L   T   F   H   L   L   R   N   M   120
361   ATC GAA ATG GCG CGA AAC GAG AAT CAA AGG GAA CAG GCG GGA CTG AAC CGC AAA TAT CTA  420
121    I   E   M   A   R   N   E   N   Q   R   E   Q   A   G   L   N   R   K   Y   L   140
421   GAT GAG GTT GGG AAG TAG ctgtagctccacgatgatgctttcaatcggcgagtaaagaacaagctcaatgcactctttcacacccccaaactctcgctt  519
141    D   E   V   G   K   *                                                          146
520   aagcatatgacattcaccgatgcctcctgctgacccacactcttagaAATAAAggttcttcagtggttccttgccactgcaatgaacctccttctcgttcttgagttggctgagg  634
635   ttccttggagATTAAAaaaggttcttcagatcaggctgttggtttttgcattccaacacaaaaaaaaaaaaaaaaaa  711
```

FIG. 2. Nucleotide and deduced amino acid sequences of the goldfish preprocorticotropin-releasing factor (A; Genbank Accession No. AF098629) and preprourotensin I (B; Genbank Accession No. AF129115) cDNAs. The DNA sequences of the 5′- and 3′-untranslated region are shown as *lower case letters,* while coding regions are shown as *upper case letters.* The putative signal peptide sequences are shown as *boldface upper case letters.* Potential enzymatic cleavage recognition sites are indicated in *black boxes.* The amino acid sequences for mature corticotropin-releasing factor and urotensin I peptides are *underlined.* The polyadenylation signals, AATAAA and ATTAAA, in the 3′-untranslated regions are shown as *upper case letters.*

TABLE 2
Percentage Amino Acid Sequence Identity of the Known Corticotropin-Releasing Factor (CRF), Urotensin I (UI), and CRF-Related Peptides and Precursors between Goldfish and Other Vertebrate Species

	Goldfish CRF		Goldfish UI	
	Peptide % identity	Precursor % identity	Peptide % identity	Precursor % identity
Goldfish CRF			53	28
White sucker CRF_1	97	91	53	29
White sucker CRF_2	95	87	53	26
Sockeye salmon CRF	78	61	56	26
Xenopus CRF	87	44	56	29
Ovine CRF	78	42	51	29
Goat CRF	78		51	
Bovine CRF	75		51	
Porcine CRF_1	95		53	
Porcine CRF_2	92		53	
Horse CRF	92		51	
Rat CRF	92	48	53	27
Human CRF	92	49	53	25
Goldfish UI	53	28		
Common carp UI	53	28	100	94
White sucker UI	53		95	
Rainbow trout UI	53	28	90	61
Flounder UI	46		68	
Sole UI	46		63	
Dogfish UI	53		53	
Rat urocortin	45	25	62	34
Human urocortin	42	26	57	27
Sauvagine	42		50	

Note. Multiple amino acid sequence comparisons were performed using CLUSTAL W (1.74) program with default parameters at the online service website of the European Bioinformatics Institute.

brain regions of goldfish (Fig. 3B). The relative regional levels of CRF mRNA in the brain of control animals was shown to be telencephalon–preoptic ≥ olfactory bulbs ≥ hypothalamus > optic tectum–thalamus ≥ posterior brain (Fig. 3D). There was no hybridization signal for CRF mRNA in the pituitary (Fig. 3D). The hybridization signals to the P7/P6 UI probe (Fig. 1) revealed a single gene transcript of approximately 2.0 kb for the UI precursor in goldfish brain (Fig. 3C). The average hybridization signal for UI mRNA in the brain of control fish was shown to be telencephalon–preoptic > optic tectum–thalamus ≥ hypothalamus > posterior brain (Fig. 3E). No hybridization signal for UI mRNA was detected in either the olfactory bulbs or the pituitary (Fig. 3E). The gene transcript for the internal control β-actin was observed in all of the tissues examined (Figs. 3B and 3C). Overall, in the different regions of the goldfish brain examined, the relative level of CRF gene expression was greater than the level of UI gene expression (Figs. 3D and 3E).

Effects of Cortisol and RU-486 on CRF and UI Gene Expression

Validation of the RIA for measuring cortisol titers in goldfish plasma was provided by the observation that the dilution curve of immunoreactive cortisol in goldfish plasma was parallel to the standard curve (Fig. 4A). Moreover, relative to the curve obtained with the cortisol standards, the antibody exhibited negligible cross-reactivity with RU-486 (Fig. 4A).

Resting plasma cortisol concentrations in the three control groups were similar and averaged approximately 34 ng/ml (Fig. 4B). Relative to the control values, plasma cortisol concentrations were significantly elevated in the sham treatment at the 6-h sampling point and returned to basal levels 24 h after the implant procedure. Fish treated with the cortisol implants were characterized by a marked elevation in plasma cortisol levels by 6 h and by a less pronounced increase after 24 h. The rapid fall in plasma cortisol between the 6- and the 24-h time points relates to the release profile of silastic implants (Pankhurst *et al.*, 1986). RU-486-treated fish exhibited high levels of plasma cortisol that were sustained throughout the sampling regime. Overall, cortisol- and RU-486-treated fish had significantly higher plasma cortisol titers than either the sham or control groups at the 6- and 24-h time points.

Under control conditions, in the fish left undisturbed, the level of CRF mRNA remained stable between the different time points in the four brain regions sampled (Figs. 5A–5D). In the sham-treated fish, while the CRF mRNA content of the olfactory bulbs, optic tectum–thalamus region, and hypothalamus did not change significantly, CRF mRNA levels in the telencephalon–preoptic region fell to 72% of control values 6 h after injection of the blank implant and recovered after 24 h. Similarly, in the cortisol-treated fish, CRF mRNA levels in the telencephalon–preoptic region were decreased to 69% of control values at the 6-h sampling point. However, unlike the sham treatment, the level of CRF mRNA in the telencephalon–preoptic region of cortisol-injected fish remained de-

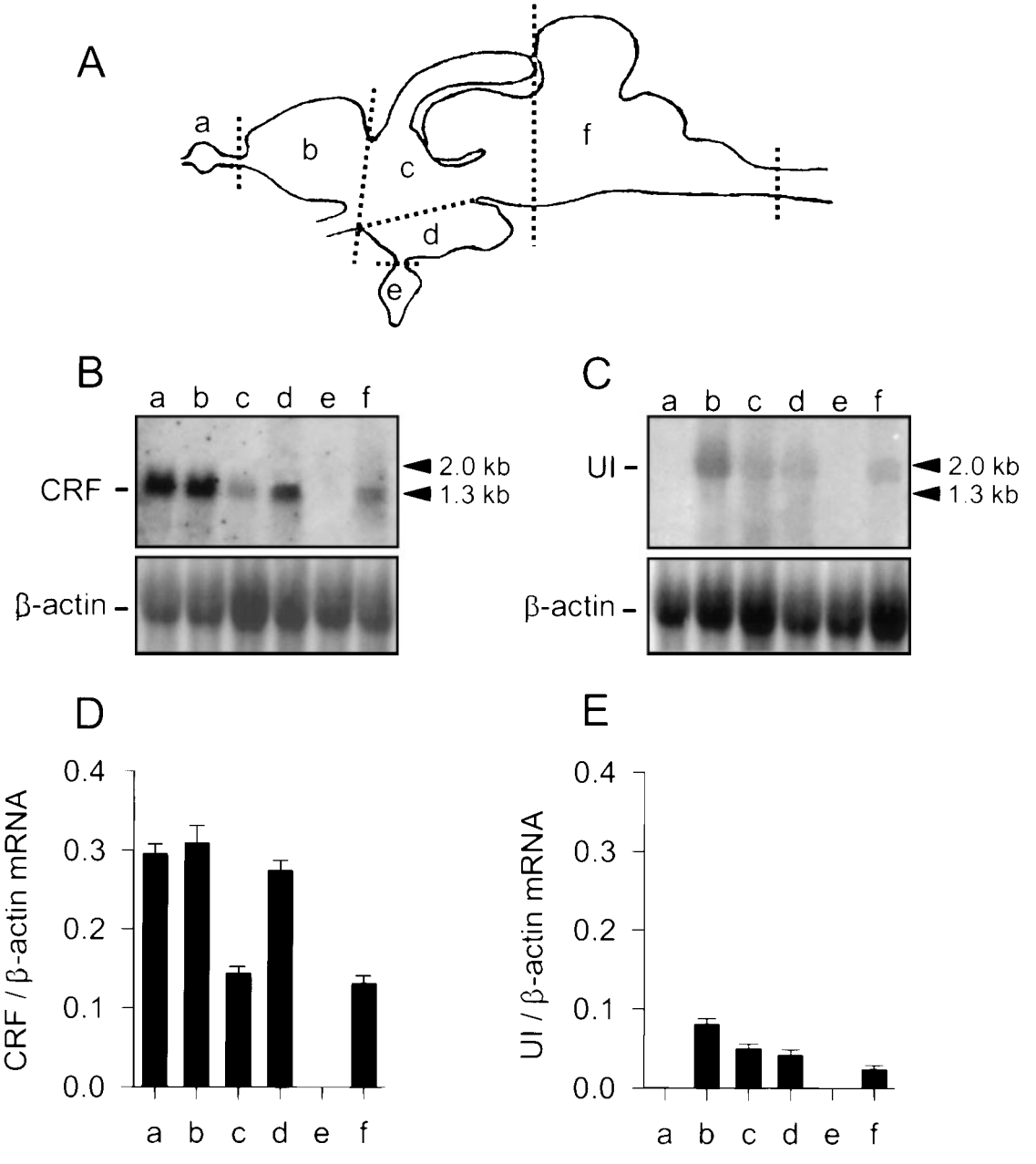

FIG. 3. Brain distribution of goldfish corticotropin-releasing factor (CRF) and urotensin I (UI) mRNAs as revealed by Northern blot analysis. (A) Dissection of the brain regions used for RNA extraction: a, olfactory bulbs; b, telencephalon–preoptic region; c, optic tectum–thalamus region; d, hypothalamus; e, pituitary; f, posterior brain (including cerebellum, medulla, and spinal cord). (B and C) Northern blot analysis of total RNA from discrete brain regions and the pituitary. RNA (20 μg) was fractionated on denaturing agorase gel and transferred onto nylon membranes. The membranes were hybridized with either ^{32}P-labeled P3 / P4 CRF probe (B) or P7 / P6 UI probe (C), exposed to a PhosphorImager screen, and quantified using ImageQuant program. Northern blot of goldfish β-actin was also performed as internal control. CRF mRNA (D) and UI mRNA (E) levels in discrete brain regions and the pituitary of goldfish ($n = 5$). Values are means + SE.

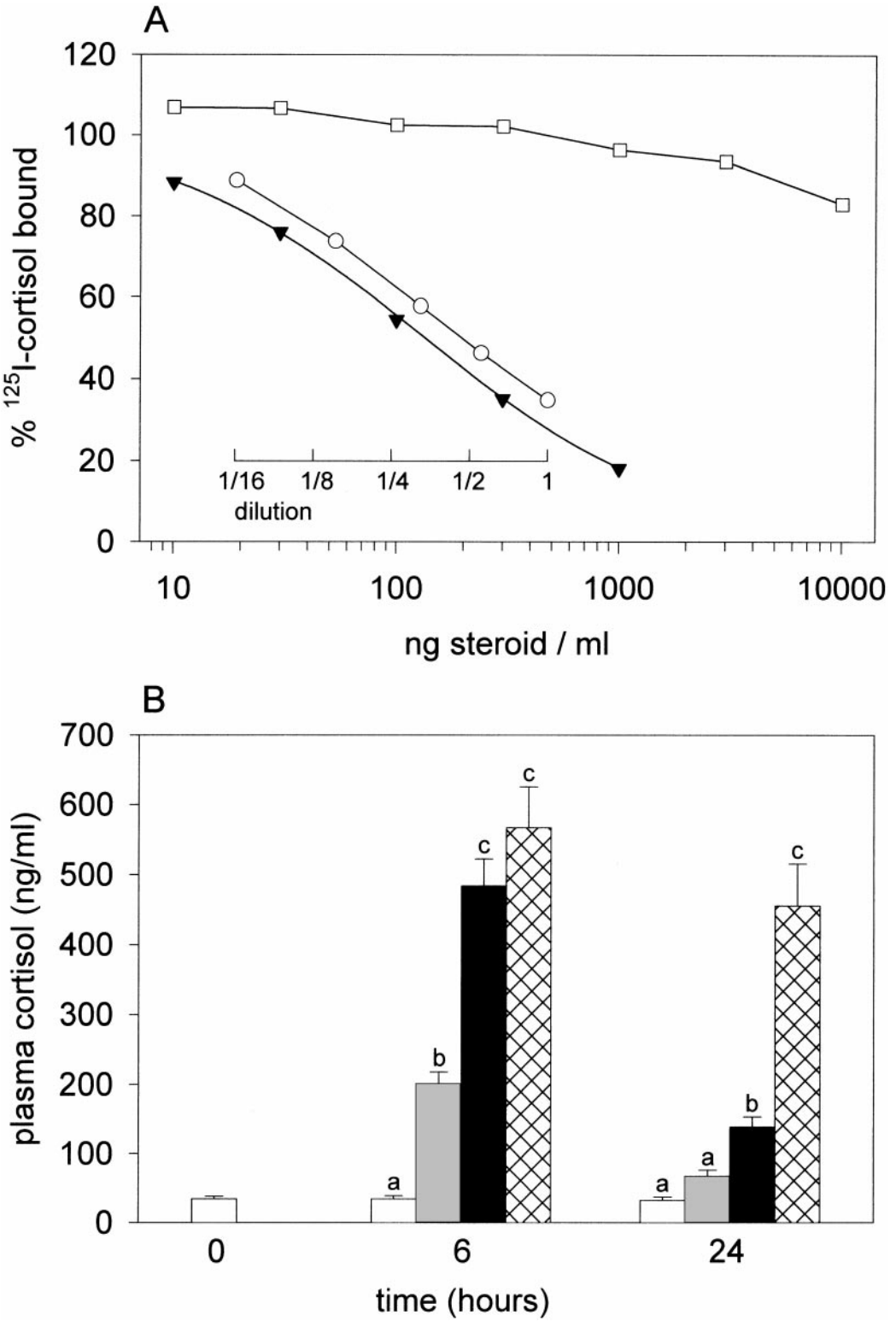

FIG. 4. (A) Standard curve for radioimmunoassay of cortisol (solid triangle), serial dilution curve of goldfish plasma (open circle), and inhibition of binding of iodinated cortisol with increasing doses of the glucocorticoid antagonist, RU-486 (open square). (B) Time course of changes in plasma cortisol in goldfish given either a sham implant (gray bars; $n = 24$), a cortisol implant (300 μg / g BW; black bars; $n = 24$), a RU-486 implant (100 μg / g BW; crosshatched bars; $n = 24$), or left undisturbed (open bars; $n = 24$). Treatments that do not share a common letter for a given time are significantly different from each other ($P < 0.05$). Values are means + SE.

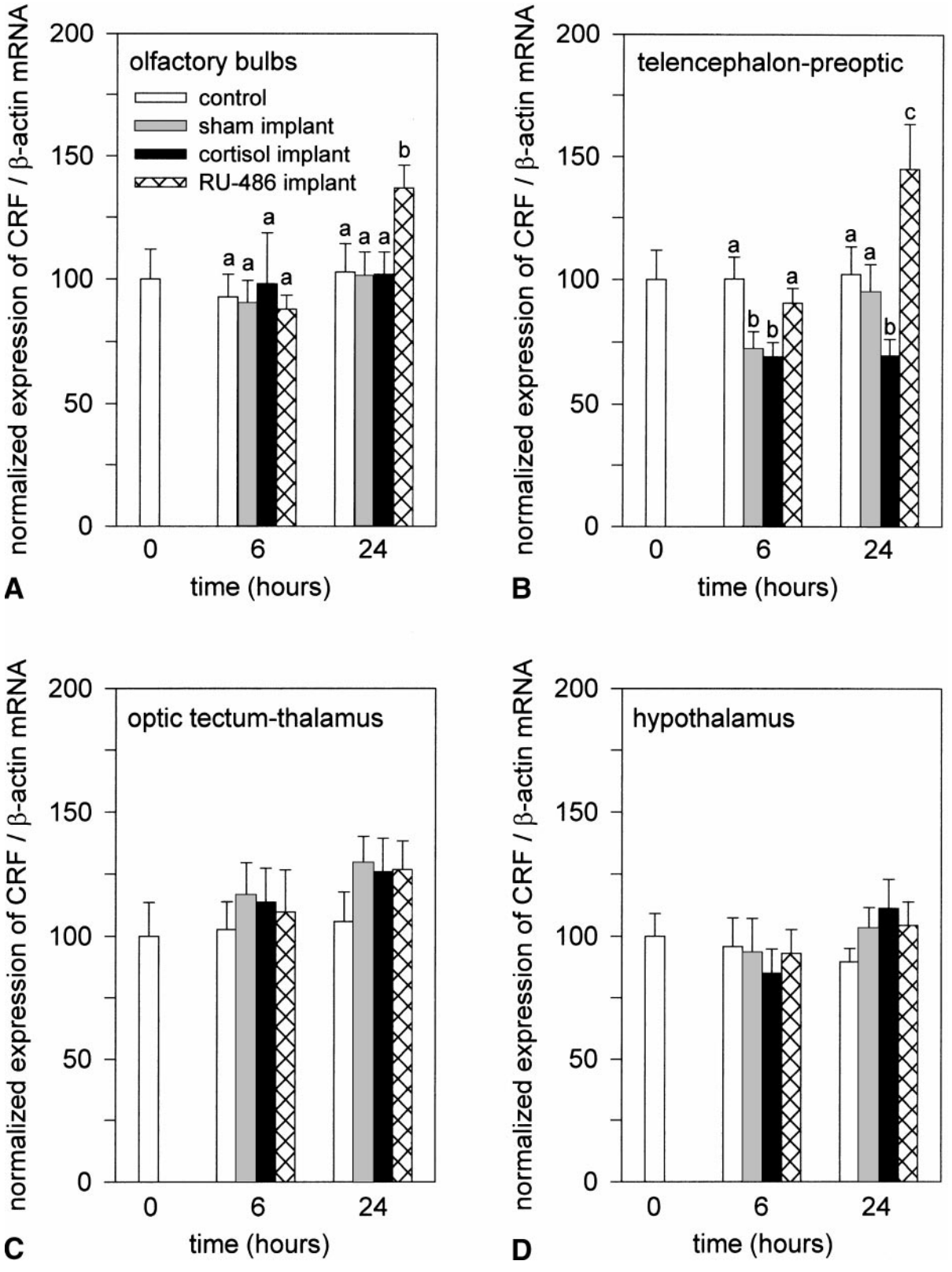

FIG. 5. Time course of changes in CRF mRNA levels in (A) the olfactory bulbs ($n = 8$), (B) the telencephalon–preoptic region ($n = 12$), (C) the optic tectum–thalamus region ($n = 12$), and (D) the hypothalamus ($n = 12$) of goldfish given either a sham implant (gray bars), a cortisol implant (300 μg / g BW; black bars), a RU-486 implant (100 μg / g BW; crosshatched bars), or left undisturbed (open bars). Treatments that do not share a common letter for a given time and brain region are significantly different from each other ($P < 0.05$). Values are means + SE.

pressed at 24 h postimplantation. In contrast, cortisol treatment did not affect CRF mRNA levels in the other three brain regions analyzed. Finally, although RU-486 treatment did not affect CRF gene expression in either the optic tectum–thalamus region or the hypothalamus, 24 h after RU-486 treatment, CRF mRNA levels increased to 137 and 145% of control values in the olfactory bulbs and the telencephalon–preoptic region, respectively.

Urotensin I mRNA levels in the telencephalon–preoptic region, the optic tectum–thalamus region, and the hypothalamus were too low to reliably assess the regulation of UI gene expression by Northern blot analysis. Therefore, we were unable to investigate the potential effects of the different implant treatments on the regulation of UI gene expression in goldfish brain.

DISCUSSION

Cloning of CRF and UI cDNAs

In the present study, we have cloned two cDNAs from a goldfish brain cDNA library that encode precursors belonging to the CRF family of peptides, preproCRF and preproUI. Both precursor polypeptides have a similar organization which is characteristic of CRF-related precursors and consist of an N-terminal signal sequence, succeeded by a divergent cryptic region, and subsequently by a conserved mature hormone sequence near the C-terminus (Fig. 1; Lederis *et al.*, 1993). However, despite these structural similarities, a comparison of the deduced amino acid sequence of the two cloned goldfish precursors and hormones with other CRF-related peptides (Table 2) shows that goldfish CRF and UI are members of two discrete lineages within the CRF family (Lovejoy, 1996; Lovejoy and Balment, 1999). In addition, similar to other CRF precursors, the cryptic region of goldfish preproCRF contains a characteristic region of conserved amino acids (residues 48–60; Fig. 2A). It has previously been suggested that this conserved region of CRF precursors might have a functional role (Morley *et al.*, 1991; Lederis *et al.*, 1993). Therefore, it is noteworthy that the same region of the three UI precursors described to date (Ishida *et al.*, 1986; Barsyte *et al.*, 1999; this study) also contains a sequence of highly conserved amino acids (residues 50–64; Fig. 2B); however, the sequence differs from that of CRF precursors.

The deduced amino acid sequence of goldfish CRF is most closely related to that of another member of the cypriniform order, the white sucker (Okawara *et al.*, 1988). In addition, goldfish CRF shares a higher percentage of sequence identity with the CRF structures of pig, horse, rat, human, and *Xenopus*, than with those of sockeye salmon and ruminant ungulates (cattle, sheep, and goats; Table 2). Relative to the rat/human CRF sequence, while sockeye salmon CRF shows 10 residue substitutions, the cyprinid CRFs have only 2–3 amino acid substitutions. The CRF structure of ruminant ungulates is thought to have undergone an accelerated rate of amino acid substitution as a result of its less dominant role in the release of ACTH (Lovejoy, 1996). Whether a similar explanation can account for the greater number of residue substitutions observed in salmon CRF than in goldfish CRF is not clear. However, there is some evidence suggesting that the role of the various corticotropin-releasing factors involved in ACTH release in salmonids and cyprinids may differ (Lederis *et al.*, 1994; Baker *et al.*, 1996).

The deduced amino acid sequence of goldfish UI is identical to the sequence of another member of the suborder cyprinidae, the common carp (Ichikawa *et al.*, 1982), and shows a high degree of sequence identity with white sucker UI (Lederis *et al.*, 1982). In contrast, the percentage identity between goldfish and dogfish UI (Waugh *et al.*, 1995) is approximately the same as between goldfish UI and other CRF-related peptides (Table 2). While the phyletic significance of this marked variation among UI peptides is not clear (Lederis *et al.*, 1993), a phylogenetic tree produced by the amino acid sequence alignment of all known CRF-related peptides (data not shown) revealed that UI peptides, urocortins, and sauvagine group together on a branch separate from the CRF peptides.

Although only one cDNA was isolated from the goldfish cDNA library for each preproCRF and preproUI, given the tetraploid nature of goldfish (Ohno *et al.*, 1967), two separate cDNAs may be expected from any given gene. Indeed, previous studies investigating tetraploid species, including white sucker (Morley *et al.*, 1991), *Xenopus* (Stenzel-Poore *et al.*, 1992), and sockeye salmon (Ando *et al.*, 1999), have all reported two types of CRF cDNAs. The sequence identity

between the two cDNAs encoding the CRF precursors within a species is high (Morley *et al.,* 1991; Stenzel-Poore *et al.,* 1992; Ando *et al.,* 1999), and Northern blot experiments have shown that the mRNAs for white sucker CRF_1 and CRF_2 are identical in size (Morley *et al.,* 1991). In goldfish, more work is needed to determine whether more than one CRF or UI precursor is expressed.

Expression Pattern of CRF and UI Genes in Goldfish Brain

Northern blot analysis of total RNA prepared from goldfish revealed a single size of CRF mRNA of approximately 1.3 kb widely distributed throughout the brain. In contrast, a similar analysis of CRF gene transcripts from white sucker hypothalamic regions revealed two mRNAs for CRF_1 and CRF_2 of 1.3 and 1.8 kb, which were differentially expressed according to the glucocorticoid status of the fish (Morley *et al.,* 1991). The two versions of CRF_1 and CRF_2 mRNA signals in white sucker are apparently derived from the same primary transcript by the use of different polyadenylation signals (Morley *et al.,* 1991). Although a previous investigation failed to detect UI transcripts in the brain of common carp (Ishida *et al.,* 1986), we observed a single size UI mRNA of approximately 2.0 kb in different regions of goldfish brain. These results concur with the single UI transcript of 2.0 kb detected in the hypothalamus of white sucker (Morley *et al.,* 1991) and contrast with the 3.2-kb UI transcript found in rainbow trout brain (Barsyte *et al.,* 1999). Since the 3′ untranslated regions of all preproUI cDNAs described to date contain multiple polyadenylation signals (Ishida *et al.,* 1986; Barsyte *et al.,* 1999; this study), the UI mRNA signals of varying size found among teleost brain are likely to be the result of differential processing of the UI transcripts. Differential processing of UI mRNA is further supported by the presence of multiple UI transcripts in the urophysis of carp (Ishida *et al.,* 1986) and trout (Barsyte *et al.,* 1999). In this study, the marked difference between the size of the cDNA (769 bp) and transcript (2.0 kb) of UI suggest that the cloned version of goldfish UI is likely a truncated transcript that was cleaved at one of the first polyadenylation signals of the 3′ terminus. Overall, in brain regions in which both UI and CRF genes are expressed, the level of hybridizable UI mRNA was three- to sevenfold lower than the level of CRF mRNA.

The detection of CRF and UI transcripts in the telencephalon–preoptic and hypothalamic regions of goldfish brain by Northern blot hybridization is consistent with the results obtained by Morley *et al.* (1991) in white sucker. Within these two brain regions, *in situ* hybridization studies have localized CRF mRNA in the NPO of white sucker and sockeye salmon (Okawara *et al.,* 1992; Ando *et al.,* 1999) and in the NPO, the ventral telencephalon, the NLT, and the nucleus recessus lateralis of goldfish (Lederis *et al.,* 1994). Overall, relative to the other brain regions analyzed, the level of CRF expression in the telencephalon–preoptic region of goldfish brain suggests, as in previous studies, that the NPO is a primary site of CRF synthesis and storage in teleosts (Lederis *et al.,* 1994). Moreover, in contrast to observations in white sucker in which UI mRNA is expressed almost equally in the NPO and the NLT (Morley *et al.,* 1991), our results suggest that the telencephalon–preoptic region is the principle site of UI synthesis in goldfish brain.

In addition to a localization of CRF and UI transcripts in the hypothalamus and telencephalon–preoptic region, our analysis of the distribution of CRF and UI synthesis in goldfish brain demonstrated that the mRNA for CRF and UI genes are expressed in extrahypothalamic regions. The presence of CRF and UI transcripts in the optic tectum–thalamus and posterior brain regions suggest that the CRF and UI peptides that have been shown immunohistochemically in these regions (Lederis *et al.,* 1985) are likely to be synthesized there. Although to our knowledge CRF peptides have not been localized in the olfactory system of fish, our results suggest that the olfactory bulbs, while devoid of UI gene expression, is the most significant extrahypothalamic site of CRF mRNA synthesis in the brain of goldfish. This observation is consistent with the extensive network of CRF-synthesizing neurons previously identified in the olfactory system of rats (Imaki *et al.,* 1989). The absence of CRF and UI gene expression in the pituitary concurs with the undetectable levels of CRF mRNA in the pituitary of rat (Jingami *et al.,* 1985). In mammals, while the CRF-synthesizing neurons that arise in the paraventricular nucleus and terminate in the median eminence control primarily the neuroendocrine component of

stress, the CRF perikarya widely distributed outside of this axis have been implicated in the autonomic and behavioral responses to stress (Turnbull and Rivier, 1997). Future investigations are needed to determine whether the extrahypothalamic CRF- and UI-synthesizing neurons identified in goldfish are also involved in mediating some of the autonomic and behavioral responses to stress.

Effects of Cortisol and RU-486 on CRF and UI Gene Expression

Results from the sham, cortisol, and RU-486 implant experiments show that stress levels of plasma cortisol can lead to a decrease in CRF gene expression that is mediated by glucocorticoid receptors in the telencephalon–preoptic region of goldfish brain. In contrast, CRF gene expression in other brain regions was unresponsive to any of the treatments. Therefore, this study provides direct evidence that cortisol is involved in the negative feedback regulation of CRF mRNA synthesis in fish and gives an indication of the regional specificity of the effects of cortisol on levels of CRF mRNA in goldfish brain.

Several studies have previously suggested that cortisol may exert negative feedback effects on the CRF-synthesizing neurons of the NPO in teleosts. For example, Fryer and Peter (1977b) showed that cortisol implants in the vicinity of the NPO in goldfish suppressed the increase in plasma cortisol that occurs following a sham-injection stress. Olivereau and Olivereau (1989) reported that CRF-like immunoreactivity in the NPO of eels (*Anguilla anguilla*) was reduced by intraperitoneal injections of cortisol. Pharmacological adrenalectomy with metopirone to suppress cortisol synthesis increased the cross-sectional area of CRF-immunoreactive neurons in the NPO of goldfish (Fryer and Boudreault-Châteauvert, 1981) and eels (Olivereau and Olivereau, 1990) and increased CRF mRNA levels in the entire hypothalamic region of white sucker (Morley *et al.*, 1991). In this study, an increase in plasma cortisol, either endogenous as a result of the sham-injection stress or exogenous via the cortisol implants, resulted in a suppression of CRF mRNA levels in the telencephalon–preoptic region of goldfish brain. Furthermore, while the temporary increase in plasma cortisol following the sham-injection stress was associated with a transient decrease in CRF gene expression, the sustained elevation in plasma cortisol in the cortisol-treated fish resulted in an extended depression of CRF mRNA levels. Since immunohistochemical and *in situ* hybridization studies have demonstrated that CRF peptide and mRNA are primarily localized in the NPO of the telencephalon–preoptic region (Lederis *et al.*, 1994), our results provide strong evidence that stress levels of plasma cortisol can inhibit CRF gene expression in the NPO of teleosts.

The results obtained with the glucocorticoid antagonist, RU-486, further support the contention that cortisol mediates the negative feedback regulation of CRF gene expression in goldfish. Although the increase in plasma cortisol 6 h after implantation of RU-486 was almost three times higher than that in the sham-treated fish, it had no effect on the levels of CRF mRNA in the telencephalon–preoptic brain region. Moreover, in the same brain region, the sustained increase in plasma cortisol 24 h after implantation of the receptor antagonist RU-486 was associated with a significant increase in CRF gene expression. Taken together, these results suggest that the negative feedback effects of cortisol on CRF gene expression are mediated by glucocorticoid receptors and can significantly dampen the stress-induced activation of the HPI axis in goldfish. In support of a glucocorticoid receptor-mediated negative feedback process, Teitsma *et al.* (1997) recently showed that glucocorticoid receptor mRNAs are heavily expressed in the NPO of rainbow trout (*Oncorhynchus mykiss*).

The stimulatory effects of RU-486 treatment on the plasma cortisol concentrations of goldfish, while in agreement with similar observations in mammalian studies, appear to be at odds with previous investigation of RU-486 effects in rainbow trout. In mammals, RU-486 administration disinhibits the HPI axis and results in a dose-dependent increase in plasma ACTH and cortisol concentrations (Gaillard *et al.*, 1984; Healy *et al.*, 1985). In contrast, RU-486 implants given intraperitoneally at a dose of 100 μg/gBW had no effect on plasma cortisol concentrations in rainbow trout (Vijayan *et al.*, 1994; Reddy *et al.*, 1995). However, while the effects of RU-486 treatment on plasma cortisol levels were assessed within 24 h of pellet implantation in this study, they were assessed on days 3 (Reddy *et al.*, 1995) and 7 (Vijayan *et al.*, 1994) postimplantation in trout. Since plasma cortisol levels return to resting

levels 3 days after RU-486 injection in goldfish (Bernier and Peter, unpublished observations), it is possible that the sampling regime used to investigate the effects of RU-486 in trout unknowingly missed the acute effects of this treatment on plasma cortisol levels.

Parallel to a cortisol-mediated suppression of CRF synthesis, several other feedback effects of cortisol on the HPI axis of goldfish may have contributed to the regulation of cortisol secretion in this study. Primary among these feedback loops is the direct inhibitory effect of cortisol on ACTH secretion from the pituitary (Fryer *et al.*, 1984). Indirect evidence also suggests that glucocorticoids inhibit the biosynthesis and release of another corticotropin-releasing peptide, arginine vasotocin (AVT), from the NPO of goldfish (Fryer and Lederis, 1988). In addition, although low mRNA levels prevented us from assessing the effects of cortisol on UI synthesis, there nevertheless is some evidence that cortisol may be involved in regulating the expression of UI in goldfish (Fryer, 1989). Therefore, the plasma cortisol levels that characterized the sham-, cortisol-, and RU-486-treated fish are likely a reflection of the activity of multiple corticotropin-releasing peptides and of multiple cortisol-mediated feedback loops.

In mammals, several studies have provided evidence for pronounced regional differences in the regulation of CRF gene expression by glucocorticoids. Results obtained by immunohistochemistry (Swanson *et al.*, 1983), Northern blot analysis, and *in situ* hybridization (Imaki *et al.*, 1991) have all shown that brain sites outside the PVN in mammals are unaffected by manipulations of glucocorticoid status. Similarly, while changes in plasma cortisol levels can alter the expression of CRF gene in the telencephalon–preoptic region of goldfish brain, they have no effect on CRF mRNA levels in the hypothalamus, the optic tectum–thalamus region, and the olfactory bulbs. In contrast, the increase in CRF gene expression in the olfactory bulbs of RU-486-treated goldfish suggest the possibility of glucocorticoid receptor-mediated CRF gene regulation in this region. However, manipulations of glucocorticoid status have no effect on CRF mRNA levels in the olfactory system of rats (Imaki *et al.*, 1991), and glucocorticoid receptors appear to be absent from the olfactory bulbs of fish (Teitsma *et al.*, 1997). These conflicting observations put into question the role that cortisol plays in the regulation of CRF gene expression in the olfactory bulb of goldfish. In general, our results indicate that cortisol does not regulate CRF gene expression in brain sites outside of the telencephalon–preoptic region of goldfish brain.

In summary, the goldfish preproCRF and preproUI cDNAs isolated in this study exhibit the characteristic structure of CRF-related precursors and the deduced amino acid sequence of their mature peptides shows that goldfish CRF and UI are members of their respective lineage within the CRF peptide family. A Northern blot analysis of CRF and UI transcripts provided evidence that both genes are differentially expressed in both hypothalamic and extrahypothalmic regions with the CRF gene having a stronger expression than the UI gene throughout goldfish brain. In the telencephalon–preoptic region, cortisol suppresses and glucocorticoid receptor blockade stimulates CRF gene expression. In contrast, cortisol does not appear to be involved in the regulation of CRF synthesis in the optic tectum–thalamus region and the hypothalamus.

ACKNOWLEDGMENTS

We acknowledge Dr. H. R. Habibi for providing us with a goldfish brain cDNA library. This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) operating grants to R.E.P. N.J.B. was the recipient of an NSERC postdoctoral fellowship.

REFERENCES

Ando, H., Hasegawa, M., Ando, J., and Urano, A. (1999). Expression of salmon corticotropin-releasing hormone precursor gene in the preoptic nucleus in stressed rainbow trout. *Gen. Comp. Endocrinol.* **113,** 87–95.

Baker, B. I., Bird, D. J., and Buckingham, J. C. (1996). In the trout, CRH and AVT synergize to stimulate ACTH release. *Regul. Pept.* **67,** 207–210.

Barsyte, D., Tipping, D. R., Smart, D., Conlon, J. M., Baker, B. I., and Lovejoy, D. A. (1999). Rainbow trout (*Oncorhynchus mykiss*) urotensin-I: Structural differences between urotensins-I and urocortins. *Gen. Comp. Endocrinol.* **115,** 169–177.

Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol.* **100,** 243–255.

Church, G., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.

Fryer, J. N. (1989). Neuropeptides regulating the activity of goldfish corticotropes and melanotropes. *Fish Physiol. Biochem.* **7,** 21–27.

Fryer, J. N., and Boudreault-Châteauvert, C. (1981). Cytological evidence for activation of neuroendocrine cells in the parvocellular preoptic nucleus of the goldfish hypothalamus following pharmacological adrenalectomy. *Cell Tissue Res.* **218,** 129–140.

Fryer, J. N., and Peter, R. E. (1977a). Hypothalamic control of ACTH secretion in goldfish: II. Hypothalamic lesioning studies. *Gen. Comp. Endocrinol.* **33,** 202–214.

Fryer, J. N., and Peter, R. E. (1977b). Hypothalamic control of ACTH secretion in goldfish: III. Hypothalamic cortisol implant studies. *Gen. Comp. Endocrinol.* **33,** 215–225.

Fryer, J. N., and Lederis, K. (1988). Comparisons of actions of posterior pituitary hormones in corticotropin secretion in mammals and fishes. *In* "Recent Progress in Posterior Pituitary Hormones" (S. Yoshida and L. Share, Eds.), pp. 337–344. Elservier, Amsterdam.

Fryer, J., Lederis, K., and Rivier, J. (1983). Urotensin I, a CRF-like neuropeptide, stimulates ACTH release from the teleost pituitary. *Endocrinology* **113,** 2308–2310.

Fryer, J., Lederis, K., and Rivier, J. (1984). Cortisol inhibits the ACTH-releasing activity of urotensin I, CRF and sauvagine observed with superfused goldfish pituitary cells. *Peptides* **5,** 925–930.

Gaillard, R. C., Riondel, A., Muller, A. F., Herrmann, W., and Baulieu, E. E. (1984). RU 486: A steroid with antiglucocorticosteroid activity that only disinhibits the human pituitary–adrenal system at a specific time of day. *Proc. Natl. Acad. Sci. USA* **81,** 3879–3882.

Healy, D. L., Chrousos, G. P., Schulte, H. M., Gold, P. W., and Hodgen, G. D. (1985). Increased adrenocorticotropin, cortisol, and arginine vasopressin secretion in primates after the antiglucocorticoid steroid RU 486: Dose response relationships. *J. Clin. Endocrinol. Metab.* **60,** 1–4.

Ichikawa, T., McMaster, D., and Lederis, K. (1982). Isolation and amino acid sequence of urotensin I, a vasoactive and ACTH-releasing neuropeptide, from the carp (*Cyprinus carpio*) urophysis. *Peptides* **3,** 859–867.

Imaki, T., Nahon, J. L., Sawchenko, P. E., and Vale, W. (1989). Widespread expression of corticotropin-releasing factor messenger RNA and immunoreactivity in the rat olfactory bulb. *Brain Res.* **496,** 35–44.

Imaki, T., Nahan, J.-L., Rivier, C., Sawchenko, P. E., and Vale, W. (1991). Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J. Neurosci.* **11,** 585–599.

Ishida, I., Ichikawa, T., and Deguchi, T. (1986). Cloning and sequence analysis of cDNA encoding urotensin I precursor. *Proc. Natl. Acad. Sci. USA* **83,** 308–312.

Jingami, H., Matsukura, S., Numa, S., and Imura, H. (1985). Effects of adrenalectomy and dexamethasone administration on the level of prepro-corticotropin-releasing factor messenger ribonucleic acid (mRNA) in the hypothalamus and adrenocorticotropin / β-lipotropin precursor mRNA in the pituitary in rats. *Endocrinology* **117,** 1314–1320.

Lederis, K., Letter, A., McMaster, D., and Moore, G. (1982). Complete amino acid sequence of urotensin I, a hypotensive and corticotropin-releasing neuropeptide from *Catostomus. Science* **218,** 162–164.

Lederis, K., Fryer, J. N., and Yulis, C. R. (1985). The fish neuropeptide urotensin I: Its physiology and pharmacology. *Peptides* **6,** 353–361.

Lederis, K. P., Ichikawa, T., Richter, D., and Schonrock, C. (1993). Molecular analysis of corticotropin-releasing factors and related peptides in teleosts. *In* "Biochemistry and Molecular Biology of Fishes" (P. W. Hochachka and T. P. Mommsen, Eds.), pp. 325–338. Elsevier, Amsterdam.

Lederis, K., Fryer, J. N., Okawara, Y., Schonrock, C., and Richter, D. (1994). Corticotropin-releasing factors acting on the fish pituitary: Experimental and molecular analysis. *In* "Fish Physiology, Volume XIII, Molecular Endocrinology of Fish" (N. M. Sherwood and C. L. Hew, Eds.), pp. 67–100. Academic Press, San Diego.

Lee, L. E. J., and Bols, N. C. (1989). The corticosteroid receptor and the action of various steroids in rainbow trout fibroblasts. *Gen. Comp. Endocrinol.* **74,** 85–95.

Lovejoy, D. A. (1996). Peptide hormone evolution: Functional heterogeneity within GnRH and CRF families. *Biochem. Cell Biol.* **74,** 1–7.

Lovejoy, D. A., and Balment, R. J. (1999). Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. *Gen. Comp. Endocrinol.* **115,** 1–22.

McMaster, D., and Lederis, K. (1988). Urotensin-I and CRF-like peptides in *Catostomus commersoni* brain and pituitary—HPLC and RIA characterization. *Peptides* **9,** 1043–1048.

Montecucchi, P. C., and Henschen, A. (1981). Amino acid composition and sequence analysis of sauvagine, a new peptide from the skin of *Phyllomedusa sauvagei. Int. J. Pept. Protein Res.* **18,** 113–120.

Morley, S. D., Schonrock, C., Richter, D., Okawara, Y., and Lederis, K. (1991). Corticotropin-releasing factor (CRF) gene family in the brain of the teleost fish *Catostomus commersoni* (white sucker): Molecular analysis predicts distinct precursors for two CRFs and one urotensin I peptide. *Mol. Mar. Biol. Biotech.* **1,** 48–57.

Ohno, S., Muramoto, J., and Christian, L. (1967). Diploid–tetraploid relationship among old-world members of the fish family *Cyprinidae. Chromosoma* **23,** 1–9.

Okawara, Y., Morley, S. D., Burzio, L. O., Zwiers, H., Lederis, K., and Richter, D. (1988). Cloning and sequence analysis of cDNA for corticotropin-releasing factor precursor from the teleost fish *Catostomus commersoni. Proc. Natl. Acad. Sci. USA* **85,** 8439–8443.

Okawara, Y., Ko, D., Morley, S. D., Richter, D., and Lederis, K. P. (1992). *In situ* hybridization of corticotropin-releasing factor-encoding messenger RNA in the hypothalamus of the white sucker, *Catostomus commersoni. Cell Tissue Res.* **267,** 545–549.

Olivereau, M., and Olivereau, J. (1988). Localization of CRF-like immunoreactivity in the brain and pituitary of teleost fish. *Peptides* **9,** 13–21.

Olivereau, M., and Olivereau, J. (1989). Quantitative changes of CRF-like immunoreactivity in eels treated with reserpine and cortisol. *Peptides* **9,** 1261–1267.

Olivereau, M., and Olivereau, J. (1990). Effect of pharmacological adrenalectomy on corticotropin-releasing factor-like and arginine vasotocin immunoreactivities in the brain and pituitary of the eel: Immunocytochemical study. *Gen. Comp. Endocrinol.* **80,** 199–215.

Pankhurst, N. W., Stacey, N. E., and Peter, R. E. (1986). An evaluation

of techniques for the administration of 17β-estradiol to teleosts. *Aquaculture* **52,** 145–155.

Peyon, P., Lin, X.-W., Himick, B. A., and Peter, R. E. (1998). Molecular cloning and expression of cDNA encoding brain preprocholecystokinin in goldfish. *Peptides* **19,** 199–210.

Reddy, P. K., Vijayan, M. M., Leatherland, J. F., and Moon, T. W. (1995). Does RU486 modify hormonal responses to handling stressor and cortisol treatment in fed and fasted rainbow trout? *J. Fish Biol.* **46,** 341–359.

Stenzel-Poore, M. P., Heldwein, K. A., Stenzel, P., Lee, S., and Vale, W. W. (1992). Characterization of the genomic corticotropin-releasing factor (CRF) gene from *Xenopus laevis:* Two members of the CRF family exist in amphibians. *Mol. Endocrinol.* **6,** 1716–1724.

Swanson, L. W., Sawchenko, P. E., Rivier, J., and Vale, W. W. (1983). Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: An immunohistochemical study. *Neuroendocrinology* **36,** 165–186.

Teitsma, C. A., Bailhache, T., Tujague, M., Balment, R. J., Ducouret, B., and Kah, O. (1997). Distribution and expression of glucocorticoid receptor mRNA in the forebrain of the rainbow trout. *Neuroendocrinology* **66,** 294–304.

Turnbull, A. V., and Rivier, C. (1997). Corticotropin-releasing factor (CRF) and endocrine responses to stress: CRF receptors, binding protein, and related peptides. *Proc. Soc. Exp. Biol. Med.* **215,** 1–10.

Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981). Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. *Science* **213,** 1394–1397.

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M. H., Lewis, K., Sutton, S., Chan, R., Turnball, A. V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P. E., and Vale, W. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* **378,** 287–292.

Vijayan, M. M., Reddy, P. K., Leatherland, J. F., and Moon, T. W. (1994). The effects of cortisol on hepatocyte metabolism in rainbow trout: A study using the steroid analogue RU486. *Gen. Comp. Endocrinol.* **96,** 75–84.

Von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14,** 4683–4690.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77,** 591–625.

Waugh, D., Anderson, G., Armour, K. J., Balment, R. J., Hazon, N., and Conlon, J. M. (1995). A peptide from the caudal neurosecretory system of the dogfish *Scyliorhinus canicula* that is structurally related to urotensin I. *Gen. Comp. Endocrinol.* **99,** 333–339.

Woo, N. Y. S., Hontela, A., Fryer, J. N., Kobayashi, Y., and Lederis, K. (1985). Activation of hypothalamo–hypophysial–interrenal system by urophysectomy in goldfish. *Am. J. Physiol.* **248,** R197–R201.

Yulis, C. R., Lederis, K., Wong, K.-L., and Fisher, A. W. F. (1986). Localization of urotensin I- and corticotropin-releasing factor-like immunoreactivity in the central nervous system of *Castostomus commersoni. Peptides* **7,** 79–86.