0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(11):4851–4860

doi: 10.1210/en.2005-0497

Urocortins of the South African Clawed Frog, *Xenopus laevis*: Conservation of Structure and Function in Tetrapod Evolution

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Several corticotropin-releasing factor (CRF) family genes have been identified in vertebrates. Mammals have four paralogous genes that encode CRF or the urocortins 1, 2, and 3. In teleost fishes, a CRF, urotensin I (a fish ortholog of mammalian urocortin 1) and urocortin 3 have been identified, suggesting that at least three of the four mammalian lineages arose in a common ancestor of modern bony fishes and tetrapods. Here we report the isolation of genes orthologous to mammalian urocortin 1 and urocortin 3 from the South African clawed frog, *Xenopus laevis*. We characterize the pharmacology of the frog peptides and show that *X. laevis* urocortin 1 binds to and activates the frog CRF_1 and CRF_2 receptors at picomolar concentrations. Similar to mammals, frog urocortin 3 is selective for the CRF_2 receptor. Only frog urocortin 1 binds to the CRF-binding protein, although with significantly lower affinity than frog CRF. Both urocortin genes are expressed in brain, pituitary, heart, and kidney of juvenile frogs; urocortin 1 is also expressed in skin. We also identified novel urocortin sequences in the genomes of pufferfish, zebrafish, chicken, and dog. Phylogenetic analysis supports the view that four paralogous lineages of CRF-like peptides arose before the divergence of the actinopterygian and sarcopterygian fishes. Our findings show that the functional relationships among CRF ligands and binding proteins, and their anorexigenic actions mediated by the CRF_2 receptor, arose early in vertebrate evolution. (*Endocrinology* 146: 4851–4860, 2005)

CORTICOTROPIN-RELEASING factor (CRF) was first isolated from the sheep hypothalamus as a potent secretagogue for pituitary ACTH (1) and has since been shown to mediate endocrine, autonomic, behavioral, and immune responses to stress (2). In nonmammalian species, CRF peptides are also potent stimulators of TSH secretion by the anterior pituitary (3). The actions of CRF are mediated by two G protein-coupled receptors designated CRF-receptor 1 (CRF_1) and CRF-receptor 2 (CRF_2) (4, 5); a third CRF receptor gene has been reported in catfish but not in any tetrapod species (6). A secreted CRF binding protein (CRF-BP) binds CRF with high affinity, thus modulating its bioavailability (7). Another soluble CRF-BP that represents a splice variant of the type 2a CRF receptor was recently isolated from mouse brain (8).

Peptides with structural similarity to CRF were discovered in fishes (urotensin I) (9) and an amphibian (sauvagine) (10) that were first considered to be orthologs of mammalian CRF. However, peptides with even greater similarity to CRF were subsequently discovered in these same vertebrate classes (11, 12). Vaughan *et al.* (13) isolated a second CRF-like peptide from rat with high sequence similarity to urotensin I and sauvagine, thus further confirming that vertebrates have at least two distinct paralogous lineages of CRF-like peptides. This novel mammalian peptide was named urocortin (now urocortin 1) (13, 14). Both CRF and urocortin 1 bind to and activate the CRF_1 and CRF_2 receptors, but CRF has higher affinity for the CRF_1 receptor, whereas urocortin 1 has higher affinity for the CRF_2 receptor (15, 16).

Recently, two other CRF-like peptides that are selective for the CRF_2 receptor were isolated by genomic analysis and subsequent molecular cloning from human and mouse (17–19) and named urocortin 2 and urocortin 3 (20). Urocortin 3 genes were also described in two species of pufferfish (*Takifugu rubripes* and *Tetraodon nigroviridis*); however, these authors did not report genes for urocortin 2 in the two fish genomes (17–19). These novel urocortins are thought to represent two other CRF-like peptide lineages, thus bringing the number of CRF paralogs in mammals to four. Recent findings show that CRF-like peptides and CRF-BPs are present in invertebrates (21, 22).

Analyses of CRF-like peptide function and evolution in vertebrates have primarily focused on two distantly related groups, the teleost fishes and the placental mammals; genes for CRF, but not urocortins, have been isolated in nonmammalian tetrapods. Here we report the molecular cloning and characterization of two CRF-like peptide genes from the South African clawed frog, *Xenopus laevis,* that are orthologous to mammalian urocortin 1 and urocortin 3. To understand the functions of urocortin 1 and urocortin 3 in frogs, we analyzed their tissue-specific expression, synthesized the deduced peptides, and determined their receptor and binding protein pharmacology. CRF peptides are known to inhibit food intake in vertebrates including frogs (23), and so we

First Published Online July 21, 2005

Abbreviations: aa, Amino acid; BW, body weight; CRF, corticotropin-releasing factor; CRF-BP, CRF binding protein; DNase, deoxyribonuclease; hCRF, human CRF; i.c.v., intracerebroventricular; K_i, inhibition constant; RACE, rapid amplification of cDNA ends; xCRF, *Xenopus* CRF.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

examined the potential for anorexigenic actions of the *X. laevis* urocortins. Through genome database searches, we also identified novel urocortin sequences from pufferfish, zebrafish, chicken, and dog. We used the deduced amino acid sequences of all known CRF-like peptides, and those that we identified in the current study, to generate gene trees to resolve evolutionary relationships among the genes.

Materials and Methods

Molecular cloning of X. laevis urocortins

We searched the *Xenopus tropicalis* genome database (assembly version 1.0; Joint Genome Institute, Walnut Creek, CA; release date, December 10, 2003) using the TBLASTN protocol with amino acid sequences of the prohormones of goldfish urotensin I (GenBank accession no. AJ005264) and human urocortin 3 (GenBank accession no. AF361943). We identified scaffolds that contained putative *X. tropicalis* genes for urotensin (urocortin) 1 and urocortin 3. We then used these sequences to design PCR primers (Table 1) to isolate full-length urocortin 1 and urocortin 3 cDNAs from *X. laevis* by RT-PCR and rapid amplification of cDNA ends (RACE) (BD Biosciences Clontech, Mountain View, CA) (24). Database searches using urotensin I or urocortin sequences failed to identify scaffolds that contained a frog urocortin 2 gene (discussed below). Signal peptide predictions were done using the SignalP program version 3.0 (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark; http://www.cbs.dtu.dk/services/SignalP/) (25).

Molecular phylogenetic analysis

We used the full-length urocortin 1 and urocortin 3 cDNA sequences from *X. laevis* to predict the mRNA sequences of the corresponding genes in *X. tropicalis.* For molecular phylogenetic analysis, we compared the sequences of the frog genes to known vertebrate CRF-like peptide genes retrieved from the European Molecular Biology Laboratory (EMBL; Heidelberg, Germany) and GenBank databases at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, MD). We also searched for pufferfish (*Takifugu rubripes, Tetraodon nigroviridis*), zebrafish (*Danio rerio*), chicken (*Gallus gallus*), and dog (*Canis familiaris*) sequences using the ENSEMBL project (Wellcome Trust Sanger Institute, Cambridge, UK). Multiple sequence alignments were conducted using ClustalW (European Bioinformatics Institute, Cambridge, UK) Phylogenetic trees were constructed on the basis of amino acid differences (p-distance) by the neighbor-joining method (26) using the software program MEGA (version 2.1; available at http://www.megasoftware.net/) (27). The reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications (28).

TABLE 1. Oligonucleotides used for the isolation of full-length cDNAs and for RT-PCR analysis of X. laevis urocortin 1 and urocortin 3

	Sequence (5′–3′)
Urocortin 1	
Cloning	
Forward	ATGAAGACTGCACTGTTGACACTGCTG
Reverse	GGGTCACTTCCCCACAGAGTCAAAA
RACE	
3′	ATGAAGACTGCACTGTTGACACTGCTG
3′-Nested	CTCAGACAGATGATAGAGATTGCC
5′	GGGTCACTTCCCCACAGAGTCAAAA
5′-Nested	GGCAATCTCTATCATCTGTCTGAG
RT-PCR	
Forward	GGGTTAAATGGGCTGTTAGGTGATG
Reverse	GGCAATCTCTATCATCTGTCTGAG
Urocortin 3	
Cloning	
Forward	CACGCTTCTCTTTTGTTCTAGGTGC
Reverse	GTGTGGAGATGGAAGGGATTTTCAC
RACE	
3′	CACGCTTCTCTTTTGTTCTAGGTGC
3′-Nested	CAGAGAGGTCTTAGAGGAGGCCA
5′	GTGTGGAGATGGAAGGGATTTTCAC
5′-Nested	ACACAGGGCCTGGCCTTCCTTAC
RT-PCR	
Forward	CAGAGAGGTCTTAGAGGAGGCCA
Reverse	TGGGCATTGGCTGCTGCTTT

Analysis of urocortin mRNA expression by RT-PCR

Total RNA was extracted from adult *X. laevis* tissues (brain, pituitary, heart, kidney, skin, and liver) and treated with deoxyribonuclease (DNase) I before reverse transcription. Equal amounts (1 μg) of DNase-digested RNA were reverse transcribed (+RT) or not (-RT) to control for genomic DNA contamination. A "hot start" protocol was used, and PCR conditions were as follows: 94 C for 4 min, 35 cycles; 94 C for 45 sec; 55 C for 45 sec; and 72 C for 1 min. Primers used for RT-PCR are given in Table 1. PCR products were analyzed on a 1.2% agarose gel containing ethidium bromide.

Peptide synthesis

X. laevis urocortin 1 and urocortin 3 peptides were synthesized by the protein structure facility at the University of Michigan (Ann Arbor, MI) on an Applied Biosystems (Foster City, CA) 433A peptide synthesizer using 9-fluorenylmethyloxycarbonyl solid-phase peptide chemistry. The peptides were amidated at the C terminus and purified by reverse-phase HPLC to greater than 90% purity.

cAMP assays

cAMP assays were conducted using HEK293 cells that were engineered to stably express *X. laevis* CRF_1, *X. laevis* CRF_2, human CRF_1, or human $CRF_{2(a)}$ receptors (15). The culture of HEK293 cells, in the presence or absence of peptides, and the determination of intracellular cAMP concentrations were conducted as described previously (16). The cAMP concentration in the supernatant was determined using the cAMP-[^{125}I] direct Biotrak assay (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. The results are presented as the mean $EC_{50}\pm$ SEM values (n = 4). Three to five independent experiments were conducted to verify the results.

Radioreceptor assays

The isolation of membranes from HEK293 cells engineered to stably express *X. laevis* or human CRF_1 or $CRF_{2(a)}$ receptors and the competition binding experiments using ^{125}I-astressin [for human CRF_1 ($hCRF_1$) and *Xenopus* CRF_1 ($xCRF_1$) receptors] and ^{125}I-antisauvagine [for $hCRF_{2(a)}$ and $xCRF_2$ receptors] were conducted as described previously (16). Radioligand binding assays were conducted in 96-well plates (Beckmann Instruments, Fullerton, CA) using a scintillation proximity assay (SPA) (29, 30). Briefly, membranes from $hCRF_1$ (5 μg), $hCRF_{2(a)}$ (1 μg), $xCRF_1$ (2.5 μg), or $xCRF_2$ (2.5 μg) receptor-expressing cells were combined with wheat germ agglutinin SPA beads (0.1–0.5 mg; Amersham) and 100 pM ^{125}I-labeled ligand. The reactions were incubated for 120 min at 22 C with shaking, followed by centrifugation to separate bound from free ligand. Nonspecific binding was determined as residual radioactivity in the presence of 1 μM human urocortin 1. Under these conditions, less than 10% of the total radioactivity was specifically bound by the different receptors.

Competitive binding assay for CRF-BP

A competitive binding/crosslinking assay was used as described previously (31, 32) to estimate inhibition constants (K_i apparent) for *X. laevis* CRF, urocortin 1, and urocortin 3 binding to the *X. laevis* CRF-BP. Ten micrograms of total brain protein were incubated with the tracer ([^{125}I]xCRF) and different concentrations of radioinert peptides (0–1 μM) before crosslinking and fractionation by SDS-PAGE. All competitive binding experiments were conducted using a pool of adult *X. laevis* brain extract (32). Binding data were analyzed by fitting the inverse hyperbolic

equation using Sigma Plot software (version 8.02; Systat Software, Inc., Richmond, CA), Statistical analysis was conducted using Student's unpaired *t* test, and the data are presented as the mean $K_{i(app)} \pm$ SEM for three replicates.

Food intake assay

All procedures were conducted in accordance with the guidelines established by the University Committee on Use and Care of Animals at the University of Michigan. We tested the effects of *X. laevis* urocortin 1 or urocortin 3 on food intake in juvenile frogs as previously described (33). In two separate experiments, animals [1.0–2.2 g body weight (BW)] that had been fasted for 48 h were assigned to one of six experimental groups (n = 4–5 per group): nonhandled control, saline-injected control, and four doses of either urocortin 1 or urocortin 3 (ng/g body weight): 0.02, 0.20, 2.0, 20.0. Frogs were anesthetized before receiving intracerebroventricular (i.c.v.) injections into the third ventricle; all animals revived within 15 min after injection. Thirty to 45 min after injection, 250 mg beef liver pieces were given to each animal; after 15 min, the remaining food was removed from the aquarium and weighed. The amount of liver eaten divided by BW (in milligrams of liver per gram of BW) was compared among treatments using ANOVA (SAS for Windows statistical software version 8; SAS Institute, Cary, NC).

Results

Molecular cloning of urocortins from X. laevis

We isolated two distinct cDNAs from *X. laevis* brain that are orthologous to two mammalian urocortin genes, and we have thus designated these *X. laevis* urocortin 1 and urocortin 3. The *X. laevis* urocortin 1 cDNA (GenBank accession no. AY596827) encodes a 158-amino-acid (aa) precursor that includes a 21-aa signal peptide (M^1-A^{21}; Fig. 1A) and a 40-aa mature peptide (E^{117}-V^{156}; Fig. 1A). The mature peptide is flanked by a dibasic cleavage site (KR) and a putative C-terminal amidation site (GK; Fig. 1A). The *X. laevis* urocortin 1 mature peptide shares sequence similarity with both fish urotensins I (54–63%) and mammalian urocortins 1 (65–70%) and 49% sequence similarity with *X. laevis* CRF. The deduced *X. laevis* urocortin 1 prohormone amino acid sequence is 86% similar to the predicted *X. tropicalis* prohormone. The mature urocortin 1 peptides of the two *Xenopus* species differ by only one amino acid in position 28 (*X. tropicalis* H *vs.* Q; supplemental Table 1, published on The Endocrine Society's Jour-

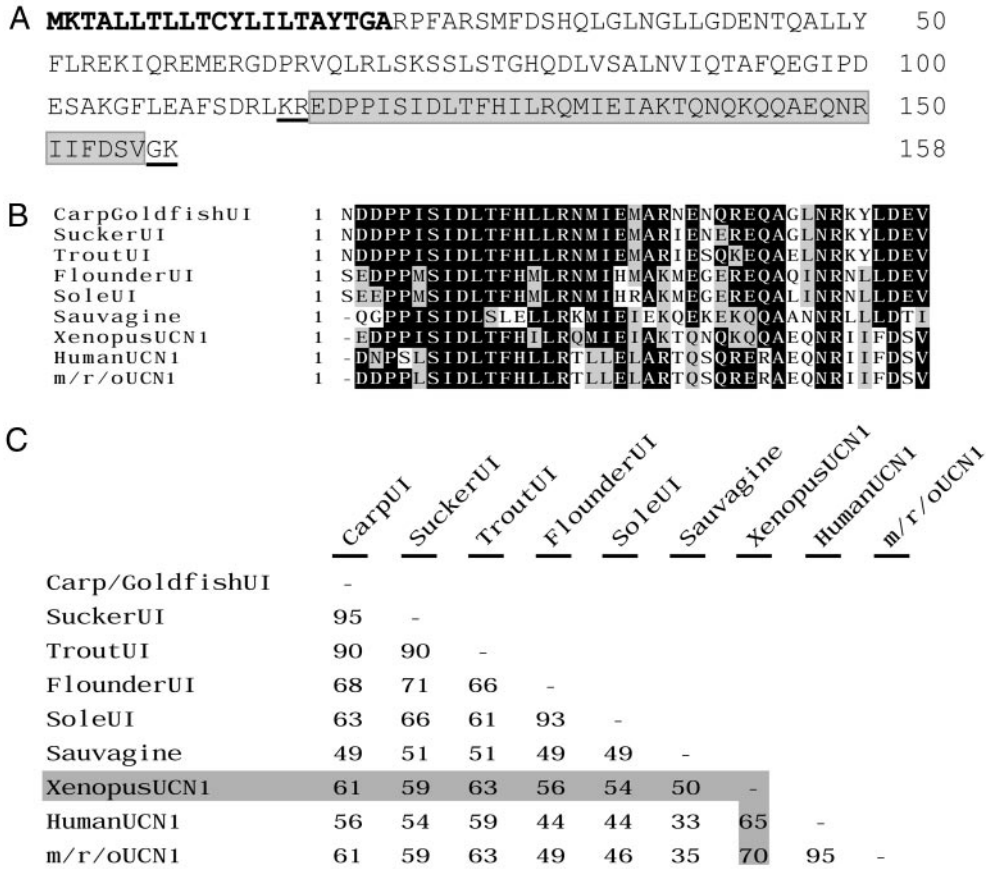


FIG. 1. Comparison of the deduced amino acid sequences of the mature peptide of *X. laevis* urocortin 1 (UCN1) and vertebrate urocortin 1 (UCN1)/urotensin I (UI)/sauvagine sequences. A, Predicted amino acid sequence encoding the *X. laevis* urocortin 1 precursor. Amino acids are numbered starting from the initiating methionine. The predicted mature peptide is indicated by the *shaded box*. Flanking the mature peptide are the predicted dibasic cleavage site and the C-terminal amidation site (*underlined*). The predicted signal peptide is indicated in *bold*. B, ClustalW alignment of mature peptide sequences for frog urocortin 1 and other urocortin 1/urotensin I/sauvagine sequences. Identical residues are indicated by *black shading*; conservative substitutions are *shaded in gray*. C, Percentages of amino acid sequence identity for urocortin 1/urotensin I/sauvagine sequences from different vertebrates. CarpGoldfish, *Cyprinus carpio, Carassius auratus*; Sucker, *Catostomus commersoni*; Trout, *Oncorhynchus mykiss*; Flounder, *Platichthys flesus*; Sole, *Hippoglossoides elassodon*, *Xenopus* (*X. laevis* and *X. tropicalis*); m/r/o, mouse/rat/ovine. See Fig. 3 legend for GenBank accession numbers.

nals Online web site at http://endo.endojournals.org). *X. laevis* urocortin 1 exhibits only 50% similarity with sauvagine (Fig. 1, B and C), a CRF-like peptide isolated from the skin of the amphibian *Phyllomedusa sauvagei* that is considered to be an amphibian UI/urocortin 1 ortholog (15). A sequence for the full-length precursor of sauvagine was recently deposited in GenBank (accession no. AY943910). The deduced amino acid sequence of the sauvagine prohormone shows that the cryptic peptide bears little or no sequence similarity to urotensins I or urocortins 1 (supplemental Fig. 1). Furthermore, the sauvagine prohormone is considerably shorter than known urotensin I or urocortin 1 prohormones (supplemental Fig. 1).

The *X. laevis* urocortin 3 cDNA (GenBank accession no. AY596826) encodes a 154-aa precursor that includes an 18-aa signal peptide (M^1–M^{18}) and a 40-aa mature peptide (T^{111}-I^{150}; Fig. 2A). A dibasic cleavage site (RR) and a putative amidation site (GRR) flank the mature peptide (Fig. 2A). The *X. laevis* urocortin 3 mature peptide bears high sequence similarity to mammalian (85–90%) and fish (73–75%) urocortins 3 (Fig. 2, B and C). The *X. laevis* urocortin 3 mature peptide has 29, 20, and 25% sequence identity with *X. laevis* CRF, *X. laevis* urocortin 1, and sauvagine, respectively. The deduced *X. laevis* urocortin 3 prohormone amino acid sequence is 91% similar to the predicted *X. tropicalis* prohormone. The mature urocortin 3 peptides of the two *Xenopus* species differ by only one amino acid in position 26 (*X. tropicalis* M *vs.* I; supplemental Table 1).

Phylogenetic analysis

The overall topology of the CRF-family tree shows at least four paralogous lineages of CRF-like peptides in vertebrates that include CRF, urocortin 1/urotensin I/sauvagine, urocortin 2, and urocortin 3 (Fig. 3, A and B; supplemental Fig. 2). To generate the most complete tree possible, we searched

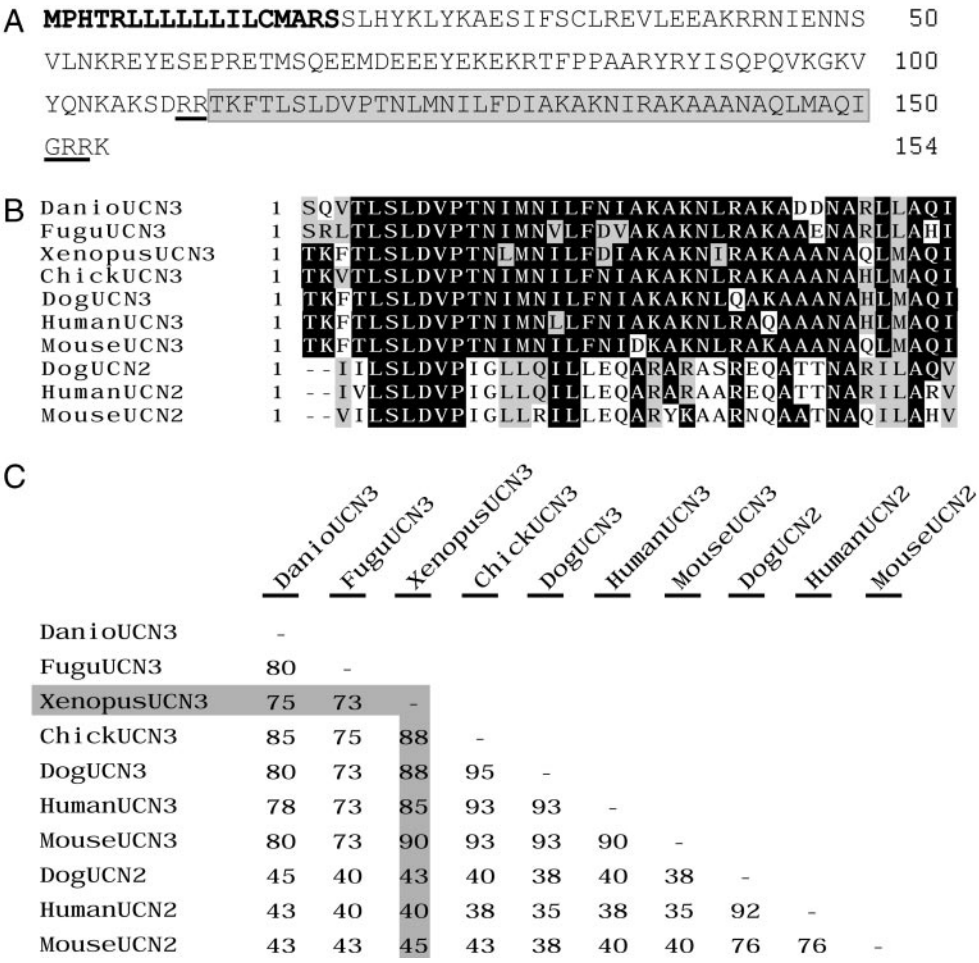
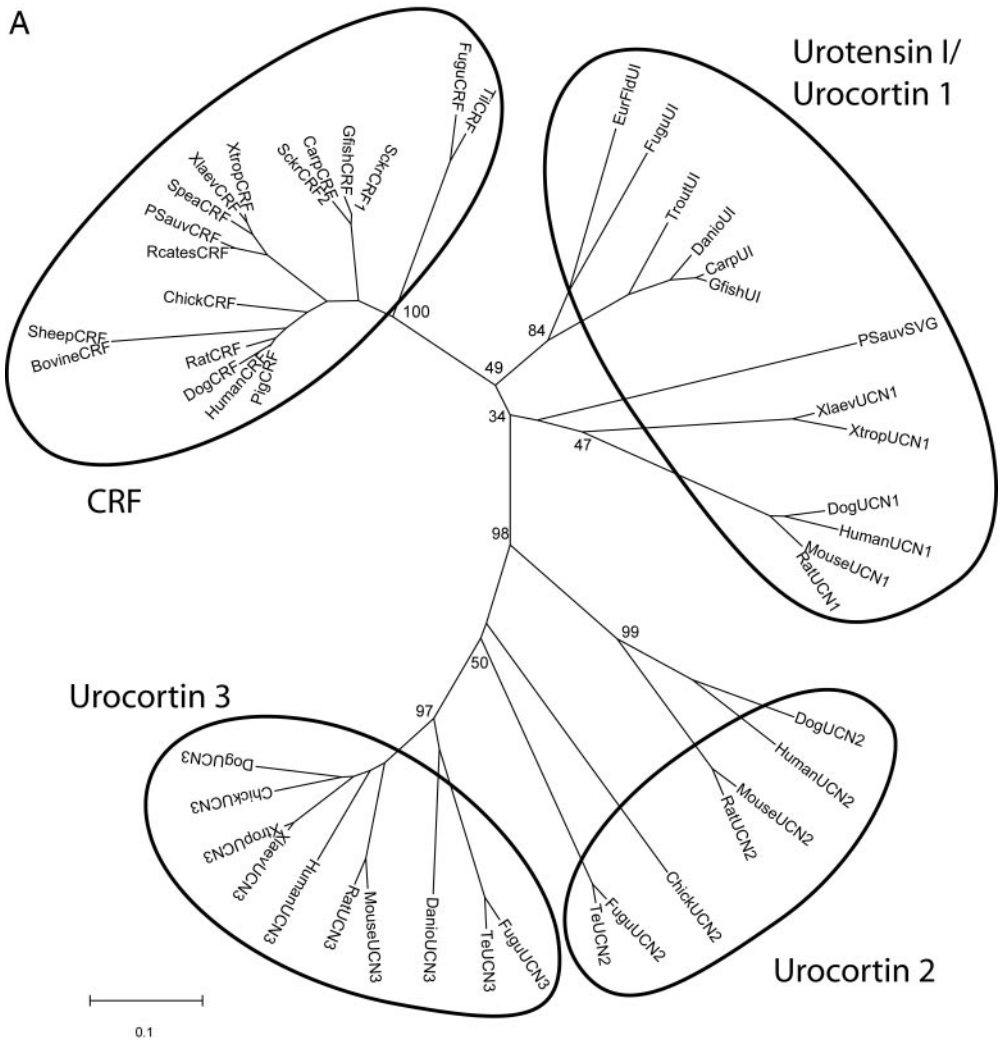


FIG. 2. Comparison of the deduced amino acid sequences of the mature peptide of *X. laevis* urocortin 3 (UCN3) and vertebrate urocortin 3 sequences. A, Predicted amino acid sequence encoding the *X. laevis* urocortin 3 precursor. Amino acids are numbered starting from the initiating methionine. The predicted mature peptide is indicated by the *shaded box*. Flanking the mature peptide are the predicted dibasic cleavage site and the C-terminal amidation site (*underlined*). The predicted signal peptide is indicated in *bold*. B, ClustalW alignment of mature peptide sequences for frog urocortin 3 and other vertebrate urocortin 3 and urocortin 2 (UCN2) sequences. Note that the dog, zebrafish (Danio), and chicken sequences were identified in the current study (supplemental Table 1). Identical residues are indicated by *black shading*; conservative substitutions are *shaded in gray*. C, Percentages of amino acid sequence identity for urocortin 3 and urocortin 2 sequences from different vertebrates. *Xenopus, X. laevis* and *X. tropicalis*; Danio, *Danio rerio*, zebrafish; Fugu, *Tetraodon nigroviridis*/*Takifugu rubripes*, pufferfish. See Fig. 3 legend for GenBank accession numbers.



B

Chromosome Location

Gene ID	Human	Chicken	Tetroadon	Fugu
TREX1	3p21.3-p21.2	12	unnamed	scaf1969
PFKFB4	3p21-p22	12	11	scaf145
UCN2	3p21.3	12	11	scaf374
Col7A1	3p21.1	12	11	not found
UQCRC1	3p21	12	11	scaf374

FIG. 3. A, Neighbor joining tree using p-distance of vertebrate CRF-like prohormone amino acid sequences. *Numbers* at branch nodes represent the confidence level of 1000 bootstrap replications. UI, Urotensin I; SVG, sauvagine; UCN1, urocortin 1; UCN2, urocortin 2; UCN3, urocortin 3; Carp, *Cyprinus carpio*; Danio, *Danio rerio*, zebrafish; EurFld, *Platichthys flesu,* European flounder; Te, *Tetraodon nigroviridi*, pufferfish; Fugu, *Takifugu rubripes*, pufferfish; Gfish, *Carassius auratus auratus*, goldfish; Xlaev, *X. laevis*, South African clawed frog; Xtrop, *X. tropicalis;* Rcates, *Rana catesbeiana*, North American bullfrog; Psauv, *Phyllomedusa sauvageii*; Sckr, *Catostomus commersoni*, sucker; Spea, *Spea hammondii*, Western spadefoot toad; Til, *Tilapia mossambicu*s; Trout, *Oncorhynchus mykiss*. GenBank accession numbers: bovine CRF, NM_001013400; carp CRF, AJ317955; carp UI, M11671; chick CRF, AJ621492; chick UCN2, XM_425157.1; chick UCN3, BX930520.2; Danio UCN3, BX004864.7; Danio UI, BX510372; dog CRF, NM_001014278; EurFld UI, AJ517171; Fugu UCN3, AJ251323.1; Gfish CRF, AF098629; Gfish UI, AF129115; human CRF, NM_000756; human UCN1, NM_003353; human UCN2, NM_033199; human UCN3, NM_053049; mouse UCN1, NM_021290; mouse UCN2, AF331517; Mouse UCN3, AF361944; pig CRF, AF440229; Psauv CRF, AY596828; Psauv SVG, AY943910; rat CRF, NM_031019; rat UCN1, NM_019150; rat UCN2, NM_133385; rat UCN3, XM_574076; Rcates CRF, AB161633; Sckr CRF1, S65264; Sckr CRF2, ×58784; Sheep CRF, J00803; Spea CRF, AY262255; Te UCN2, AL175143; Til CRF, AJ011835; Trout UI, AJ005264; Xlaev CRF, S50096; Xlaev UCN1, AY596827; Xlaev UCN3, AY596826. ENSEMBL gene ID numbers are as follows: dog UCN1, ENSCAFG00000004852; dog UCN2, ENSCAFG00000012466; dog UCN3, ENSCAFG00000005250; Fugu CRF, SINFRUG00000146091; Fugu UI, SINFRUG00000137751; Te UCN3, GSTENG00027885001; Xtrop CRF, ENSXETG00000020294; Xtrop UCN3, ENSXETG00000016289. B, Synteny mapping of urocortin 2 genes of human, chicken, and two pufferfish species. TREX1, three prime repair exonuclease 1 (AF151105); PFKFB4, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (AY786551); UCN2, urocortin 2 (AF320560); Col7A1, collagen VII, α-1 polypeptide (NM_000094); UQCRC1, ubiquinol-cytochrome c reductase core protein I (NM_003365). The Fugu UCN2 sequence is from scaffold 374 of the *Takifugu rubripes* genome project (assembly 2.0). The Xtrop UCN1 sequence is from scaffold 39327 of the *X. tropicalis* genome project (assembly 1.0).

GenBank and several independent genome databases for CRF-like peptides from each paralogous lineage. The CRF sequences form a distinct lineage, and branching corresponds to the different vertebrate classes for which sequences are available. Fish urotensin I and mammalian urocortin 1 cluster together and are separated by the *X. laevis* and *X. tropicalis* urocortin 1 sequences that we have identified, reflecting their proposed orthology. Sauvagine also lies within this cluster, but its primary sequence is the least conserved among this group of peptides. The sauvagine prohormone sequence groups closest to the frog urocortins 1. However, its inclusion in the tree destabilizes the urocortin 1 and urotensin I clade (see weak bootstrap values in the tree), with the result that these sequences fail to form a monophyletic group. Removal of the sauvagine sequence results in a bootstrap value of 99%.

The *X. laevis* and *X. tropicalis* urocortin 3 prohormones and mature peptides cluster with the mammalian and fish sequences (Fig. 3A; supplemental Fig. 2). Through genome database searches, we also identified novel sequences in dog, chicken, zebrafish, and pufferfish (*Tetraodon*) that group with vertebrate urocortin 3 genes (Figs. 2 and 3; supplemental Table 1).

Urocortin 2 genes have thus far been identified and characterized only in mammals, and these genes form a group that branches from the urocortin 3 lineage (Fig. 3A). We were unsuccessful in identifying similar sequences in the *X. tropicalis* genome, probably because the sequencing of this genome is not complete. We also failed to isolate frog urocortin 2 sequences by degenerate RT-PCR (data not shown). However, we searched several genome databases and identified a urocortin 2 gene in dog and candidate urocortin 2 genes in the chicken and pufferfish genomes (both *Takifugu* and *Tetraodon*) that share sequence similarity with mammalian urocortin 2 and vertebrate urocortins 3 (these novel chicken and pufferfish sequences are labeled urocortin 2 in Fig. 3A; supplemental Table 1). Phylogenetic analysis failed to clearly place the chicken and pufferfish sequences to either the urocortin 2 or urocortin 3 clade. However, we conducted synteny mapping of genes neighboring human urocortin 2, and this clearly showed that these sequences are in fact chicken and pufferfish orthologs of mammalian urocortin 2 (Fig. 3B).

Two pufferfish genes were previously identified as urocortin 3 (17, 19). Our phylogenetic analysis supports placing the *Takifugu* sequence (accession no. AJ251323) within the urocortin 3 clade. However, the putative *Tetraodon* urocortin 3 sequence (accession no. AL175143) reported by Hsu and Hsueh (17) shares less sequence similarity with mammalian, amphibian, and other fish urocortin 3 sequences identified here and previously. Instead, based on synteny analysis of this gene, and the fact that we have identified a distinct *Tetraodon* gene whose mature peptide is identical to the *Takifugu* urocortin 3 (Fig. 3B; supplemental Table 1), we conclude that this *Tetraodon* sequence should be named *Tetraodon* urocortin 2.

Tissue distribution of urocortin mRNAs in the frog

We analyzed the distribution of *X. laevis* urocortin 1 and urocortin 3 mRNAs in select tissues of the frog by RT-PCR. Urocortin 1 mRNA was detected in brain, pituitary, heart, kidney, and skin but not in the liver (Fig. 4). Urocortin 3 mRNA was detected in brain, pituitary, heart, and kidney but not in skin or liver (Fig. 4).

Stimulation of intracellular cAMP by CRF-like peptides

The *X. laevis* urocortin 1 increased cAMP accumulation in HEK-293 cells expressing the *X. laevis* CRF_1 receptor with potency in the picomolar range (Table 2). The potency of urocortin 1 was approximately 3 times greater on the CRF_2 receptor-expressing cells compared with the CRF_1 cells. By comparison, *X. laevis* CRF exhibited approximately 2.5 times greater potency than urocortin 1 on CRF_1 cells, but had approximately 12.5 times lesser potency on CRF_2 cells.

The *X. laevis* urocortin 3 exhibited very low potency in CRF_1 receptor-expressing cells (~3300 times less than urocortin 1). By contrast, *X. laevis* urocortin 3 exhibited moderate potency in CRF_2 cells (which was approximately 6 times less than urocortin 1, but two times greater than CRF). Thus, the potency relationships for the *X. laevis* CRF-like peptides on the frog CRF receptors were as follows: CRF>urocortin 1⋙urocortin 3 for *X. laevis* CRF_1 receptor-expressing cells; urocortin 1>urocortin 3>CRF for *X. laevis* CRF_2 receptor-expressing cells.

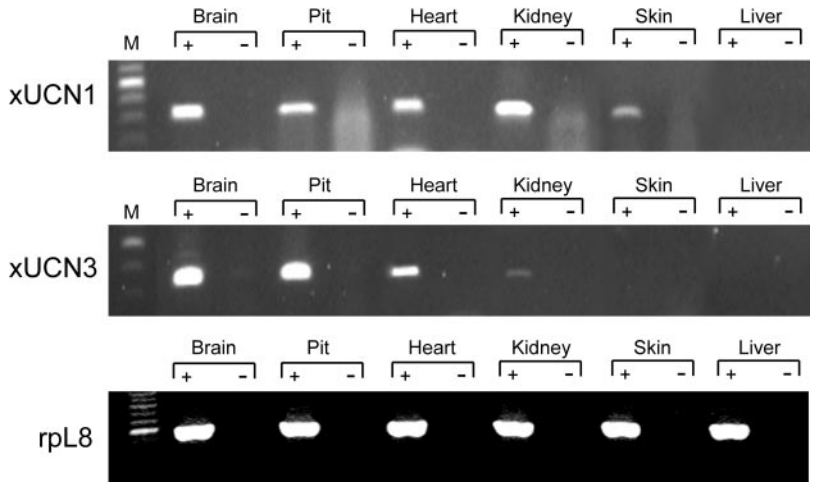


FIG. 4. RT-PCR analysis of urocortin 1 (A) and urocortin 3 (B) mRNA expression in different tissues of adult *X. laevis*. Two micrograms total RNA from brain, pituitary (pit), heart, kidney, skin, and liver was first treated with RNase-free DNase and then reversed transcribed (RT +) or not (RT -) before PCR amplification and fractionation on a 1.2% agarose gel. The rpL8 is a housekeeping gene.

TABLE 2. Functional activities and binding properties of frog CRF-like peptides

Ligand	CRF_1		CRF_2		CRF-BP
	EC_{50} (nM)	K_i (nM)	EC_{50} (nM)	K_i (nM)	K_i (nM)
Urocortin 1	0.157 ± 0.058	5.42 ± 0.79	0.049 ± 0.018	1.91 ± 0.18	50.31 ± 2.68
Urocortin 3	524 ± 89	3900 ± 1050	0.285 ± 0.04	8.32 ± 0.95	>1 μM
CRF	0.061 ± 0.009	ND	0.613 ± 0.105	ND	4.07 ± 0.27
Sauvagine	2.41 ± 0.59	1050 ± 180	0.004 ± 0.0005	2.81 ± 0.63	ND

Experiments for CRF receptors were conducted on HEK293 cells stably expressing *X. laevis* CRF_1 or $CRF_{2(a)}$ receptors. The cAMP data are representative of three to five independent stimulations performed in quadruplicate. Binding data are representative of three independent binding experiments performed in triplicate using ^{125}I-astressin (CRF_1) or ^{125}I-antisauvagine (CRF_2) as radiolabel. Binding affinity for the frog CRF-BP was determined on brain extracts using a crosslinking/competitive binding assay with [^{125}I]xCRF as tracer. ND, Not determined.

Sauvagine had approximately 15 times lesser potency than *X. laevis* urocortin 1 on CRF_1 receptor expressing cells but was the most potent peptide tested on the CRF_2 cells (~12 times more potent than *X. laevis* urocortin 1). Comparisons of homologous and heterologous peptides activating cells expressing the human CRF_1 and $CRF_{(2a)}$ receptors, or heterologous peptides activating cells expressing the frog receptors, are given in supplemental Table 2.

Radioreceptor assays

Radioreceptor assays using membranes isolated from HEK293 engineered to stably express the *X. laevis* CRF_1 or CRF_2 receptors were used to estimate K_i values for the *X. laevis* urocortins (Table 2). The *X. laevis* urocortin 1 bound with highest affinity to the CRF_2 receptor, which was approximately 3 times greater than binding to the CRF_1. This relationship is paralleled by the relative potencies of these two peptides in the cAMP accumulation assays described above.

The *X. laevis* urocortin 3 exhibited very low, micromolar affinity for the CRF_1 receptor but bound to the CRF_2 receptor with moderate affinity (~4.5 times lower than urocortin 1.) Again, these receptor binding data parallel the potency relationships obtained in the cAMP accumulation assays described above. Comparisons of homologous and heterologous peptides binding to the human CRF_1 and $CRF_{(2a)}$ receptors, or heterologous peptides binding to the frog receptors, are given in supplemental Table 3.

Competitive binding assays for CRF-BP

The CRF-BP bound urocortin 1 with considerably lower affinity than CRF (~12 times; Table 2). Urocortin 3 did not displace the [^{125}I]xCRF label from the CRF-BP even at the highest dose tested (1 μM). A panel of radioinert CRF-like peptides (frog CRF, fish urotensin I, sauvagine, frog and rat urocortins 1, and mouse urocortin 2) all competed to varying degrees for [^{125}I]xCRF binding to the CRF-BP at the two doses tested (20 and 100 nM; Fig. 5). By contrast, neither *X. laevis* nor mouse urocortins 3 competed for [^{125}I]xCRF binding at any dose tested (Fig. 5).

Food intake assays

Injections (i.c.v.) of *X. laevis* urocortin 1 or urocortin 3 suppressed food intake, and urocortin 1 was more potent than urocortin 3 (Fig. 6). Urocortin 1 exhibited a dose-dependent suppression of food intake beginning at 0.2 ng/g BW (ANOVA, $F_{(6, 26)} = 26.46$; $P = 0.0001$). By contrast, urocortin 3 suppressed food intake only at the highest dose tested (20 ng/g BW; ANOVA urocortin 3, $F_{(6, 21)} = 3.35$; $P = 0.018$).

Discussion

This is the first report of the molecular cloning of urocortin genes from a nonmammalian tetrapod. We isolated and characterized two urocortins from the South African clawed frog *X. laevis*, one orthologous to mammalian urocortin 1/fish urotensin I and the other to mammalian/fish urocortin 3. The deduced *X. laevis* urocortin 1 mature peptide is 40 aa in length and shares 70% sequence similarity with mouse/rat urocortin 1 and 63% similarity with trout urotensin I (Fig 1, B and C). Interestingly, the *X. laevis* urocortin 1 mature peptide shares only 50% sequence similarity with sauvagine, a peptide isolated from the skin of the frog *P. sauvagei* that is thought to represent an amphibian ortholog of fish urotensins 1 and mammalian urocortins 1 (10); and sauvagine shares only 35% and 49% sequence similarity with rodent urocortin 1 and trout urocortin I, respectively. A full-length sauvagine cDNA sequence isolated from the skin of *P. sau-*

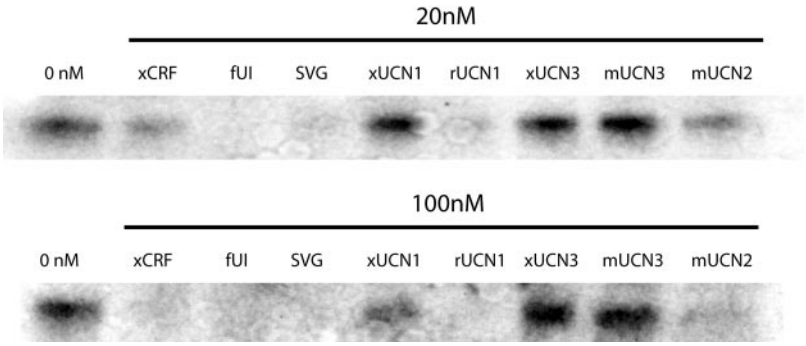


FIG. 5. Displacement of [^{125}I]-×CRF binding to CRF-BP in *X. laevis* brain extract by different radioinert peptides. Fifteen thousand cpm of [^{125}I]xCRF and 20 nM (*top panel*) or 100 nM (*bottom panel*) of each radioinert peptide were incubated with 10 μg brain protein before crosslinking and analysis by SDS-PAGE and autoradiography (see *Materials and Methods*). f, fish; r, rat; m, mouse; UI, urotensin I; SVG, sauvagine; UCN, urocortin.

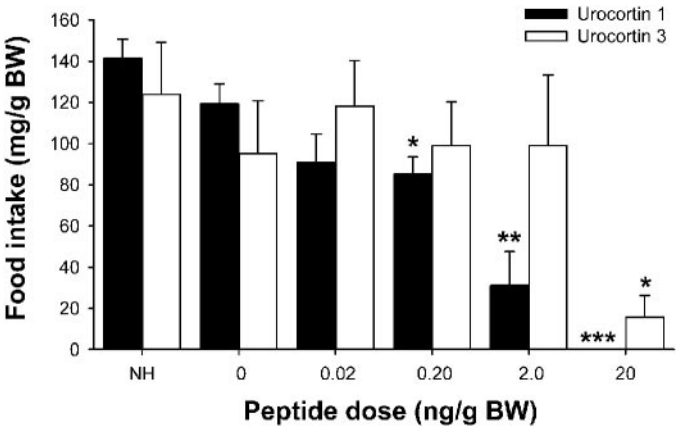


FIG. 6. Effects of urocortin 1 and urocortin 3 on food intake in *X. laevis* juveniles. Peptides were injected i.c.v., and food intake was measured. *Bars* represent mean + SEM of food eaten in a 15-min trial. *Asterisks* designate treatments that are significantly different from the saline control (Tukey tests, $\alpha = 0.05$); *multiple asterisks* distinguish significantly different treatment means among doses of urocortin 1.

vagei was recently reported (GenBank accession no. AY943910). Sauvagine appears to be a highly divergent urocortin 1, perhaps specific to *P. sauvagei*. We isolated a full-length CRF cDNA from *P. sauvagei* (accession no. AY596828; Boorse, G. C., and R. J. Denver, unpublished data), and the mature peptide is identical to two other amphibian CRFs (*X. laevis* and *Spea hammondii*). Molecular cloning of urocortins from *P. sauvageii* and other species will be required to resolve the phylogenetic relationships among these genes in the Amphibia.

We named the other *X. laevis* peptide urocortin 3 based on its high sequence similarity to urocortins 3 that have been described in fishes and mammals (17, 19). This peptide is remarkably conserved, with up to 90% sequence similarity with mammalian and 75% sequence similarity with fish urocortins 3. By genome analysis, we also identified urocortin 3 orthologs from chicken and zebrafish, which further supports the conclusion that a urocortin 3-like peptide gene was present in ancestors of the sarcopterygian and actinopterygian fishes and is thus likely to be present in all tetrapod classes. The urocortin 3 protein has not been isolated from any species, but cDNAs from mouse and human were reported by two groups who each predicted different lengths for the mature peptides (17, 19). Lewis *et al.* (19) predicted a 38-aa mature peptide, whereas Hsu and Hsueh (17) predicted a 40-aa mature peptide with two additional amino acids (TK) at the N terminus. These different predictions arose from the lack of conservation in the putative N terminal dibasic cleavage site in the rodent and human precursors (Fig. 7). However, the urocortins 3 of frog, chicken, dog, and fishes have conserved N-terminal dibasic cleavage sites (RR). This leads to the prediction that TK are the first two residues of the mature peptide in frog, chicken, and dog, and S(R/Q) in the fishes (Fig. 7). Thus, the urocortin 3 mature peptides in these species are likely to be 40 aa in length.

Although it is clear that the CRF, urocortin 1, and urocortin 3 genes arose before the divergence of the lineages that gave rise to the modern bony fishes (Teleostei) and the tetrapods, the phylogeny of urocortin 2 genes has been uncertain because urocortin 2 was cloned from only mouse and human. In searching the pufferfish and chicken genomes, we identified novel DNA sequences that encode proteins with sequence similarity to both urocortin 3 and urocortin 2 (see also Ref. 34). These sequences are distinct from the pufferfish and chicken urocortin 3 genes (described above) but are sufficiently divergent from mammalian urocortin 2 that we were unable to place them within a clade. We hypothesized that these novel sequences represent urocortin 2 genes, and to test this we conducted synteny mapping using human urocortin 2 as the landmark gene. We found that each species has homologous neighboring genes to those that we predict are urocortin 2. Based on this finding, we conclude that these pufferfish and chicken sequences are orthologs of mammalian urocortin 2. We were unable to identify similar urocortin 2 sequences by searching the *X. tropicalis* genome or by degenerate RT-PCR in *X. laevis* (data not shown). However, we predict that frogs (and all tetrapod classes) have a urocortin 2 gene that will be identified as the frog genome project reaches completion.

Our expression analyses found urocortin 1 and urocortin 3 mRNAs in the central nervous system and in several peripheral tissues of *X. laevis*. These genes are expressed in the same tissues in frogs as in mammals (13, 35–37). Earlier we found that CRF receptors are expressed in these same tissues in frogs (38). Locally produced CRF or urocortins acting via paracrine and/or autocrine pathways have been implicated in diverse physiological effects in peripheral tissues in mammals (39–41). Thus, the expression of urocortins in peripheral tissues of the frog suggests that local paracrine actions of CRF peptides arose early in vertebrate evolution.

The specificity and affinity relationships among CRF ligands and their binding proteins are phylogenetically ancient. For example, we found that frog CRF activates the frog CRF_1 receptor with greater potency than urocortin 1 but that these potency relationships were reversed for the frog CRF_2

FIG. 7. ClustalW alignment of amino acid sequences of vertebrate urocortins 3. Potential dibasic cleavage sites are indicated by *solid black* or *gray boxes* (predicted by Lewis *et al.* in Ref. 19). The mature peptide sequence is represented by corresponding *black* or *gray open boxes*.

receptor. In mammals, a similar situation exists that has led to the hypothesis that the CRF_2 receptor is a urocortin receptor. Further support for this idea stems from the finding that urocortin 2 and urocortin 3 are selective CRF_2 receptor agonists (17, 19). We found a similar selectivity for frog urocortin 3 on the frog CRF_2 receptor. In support of the data that we obtained in HEK-293 cells engineered to stably express frog CRF receptors, we found similar results in XLT-15 cells, a *X. laevis* tadpole tail muscle-derived cell line (42) that expresses native CRF_1 but not CRF_2 or CRF-BP (38). *X. laevis* CRF increased cAMP accumulation in XLT-15 cells with three times the potency of *X. laevis* urocortin 1 and 20 times the potency of sauvagine (data not shown). By contrast, both *X. laevis* and mouse urocortins 3 exhibited very low potency, with only the highest dose tested (500 nM) producing a small elevation in cAMP.

We also found that the overall specificity and affinity relationships between CRF ligands and the CRF-BP in frogs are similar to those reported in mammals, although with two notable exceptions. The frog CRF-BP had the greatest affinity for CRF, whereas the affinity for frog urocortin 1 was 10-fold lower. This was surprising, because we had previously shown that rat urocortin 1 bound the frog CRF-BP with roughly equal affinity to CRF, which is also the case for the CRF-BP in mammals (32). The frog urocortin 3 did not compete for binding to the frog CRF-BP, which is similar to the situation in mammals (19, 43). Thus, the frog CRF-BP preferentially binds frog CRF with much lower or no affinity for the frog urocortins. We tested mouse urocortin 2 and found that it competed for [^{125}I]xCRF binding to the frog CRF-BP at the two doses tested (20 nM and 100 nM; Fig. 5). Whether a similar relationship exists for a frog urocortin 2 and the CRF-BP, or if this result is simply an artifact of heterologous protein interactions, remains to be determined. Lewis *et al.* (19) reported that urocortin 2 (mouse or human) did not bind to the human CRF-BP, whereas Jahn *et al.* (43) recently reported that mouse urocortin 2 bound with high affinity to the rat CRF-BP.

Urocortins (and CRF) are known to be potent anorectic agents and are hypothesized to play an important role in suppressing appetite, especially during the fight-or-flight response (44–46). We also found that, in frogs, both urocortin 1 and urocortin 3 inhibited food intake when injected into the third ventricle (see Fig. 6); earlier we found similar appetite suppressive actions of frog CRF (33). It is noteworthy that the minimum effective dose for urocortin 1 in this study was 10 times lower than what we found was effective for CRF in a previous study (33). Similarly, urocortin 1 and urotensin I were found to be more potent appetite inhibitors than CRF in mammals (47, 48) and fish (49), respectively, and the minimum effective dose for frog urocortin 1 in the current study was similar to that found for mammals and fish (49, 50). In mammals, the appetite suppressive actions of CRF-like peptides are thought to be mediated by the CRF_2 receptor (51). The greater potency of urocortin 1 compared with CRF on food intake in mammals is attributed to its higher affinity for the CRF_2 receptor than that of CRF (48), and we conclude a similar explanation in the frog.

In comparing actions of the urocortins in the frog, we found that urocortin 1 was more potent than urocortin 3 in inhibiting appetite, which reflects differences in their affinities/potencies on the CRF_2 receptor (4- to 6-fold; Table 2). The fact that urocortin 3 suppressed appetite confirms that the anorectic action of CRF-like peptides in the frog is mediated, at least in part, by the CRF_2 receptor. Similar anorectic actions of urocortin 3 have been reported in rats (46). Our findings suggest that the regulation of food intake via the CRF_2 receptor is evolutionarily conserved among tetrapods.

In summary, we have identified orthologs of mammalian urocortin 1 and urocortin 3 in the South African clawed frog *X. laevis*. Our findings show that the CRF-like peptide genes, and the relationships among CRF ligands and their binding proteins, have been maintained through natural selection, owing to the critical role that these peptides play in development, homeostasis, and behavior. Furthermore, our analyses support the conclusion that at least four paralogous lineages of CRF peptide genes were present before the divergence of the sarcopterygian and actinopterygian fishes and have been maintained in extant vertebrate species.

Acknowledgments

Drs. Wylie Vale and Jean Rivier kindly provided synthetic *X. laevis* CRF and sauvagine. The XLT-15 cells were a kind gift of Dr. Yoshio Yaoita. We are grateful to three anonymous reviewers for their comments on the manuscript.

Received April 26, 2005. Accepted July 14, 2005.

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This work was supported by National Science Foundation Grants IBN9974672 and IBN0235401 (to R.J.D.). G.C.B. was supported by a National Science Foundation predoctoral fellowship. This work used the Molecular Core of the Michigan Diabetes Research Training Center funded by National Institutes of Health 5P60 DK20572 from the National Institute of Diabetes and Digestive Kidney Disease.

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