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# Corticotropin-Releasing Factor Is Cytoprotective in *Xenopus* Tadpole Tail: Coordination of Ligand, Receptor, and Binding Protein in Tail Muscle Cell Survival

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**Upon metamorphosis, amphibian tadpoles lose their tails through programmed cell death induced by thyroid hormone ($T_3$). Before transformation, the tail functions as an essential locomotory organ. The binding protein for the stress neuropeptide corticotropin-releasing factor (CRF; CRF-BP) is strongly up-regulated in the tail of *Xenopus* tadpoles during spontaneous or $T_3$-induced metamorphosis. This finding led us to investigate physiological roles for CRF and CRF-BP in tadpole tail. We found CRF, CRF-BP, and functional $CRF_1$ receptor in tail and CRF and functional $CRF_1$ receptors, but not CRF-BP, in the tail muscle-derived cell line XLT-15. CRF, acting via the $CRF_1$ receptor, slowed spontaneous tail regression in explant culture and caused a reduction in caspase 3/7 activity. CRF increased, but stable CRF-BP overexpression decreased, [$^3$H]thymidine incorporation in XLT-15 cells. Overexpression of CRF-BP *in vivo* accelerated the loss of tail muscle cells during spontaneous metamorphosis. Lastly, exposure of tail explants to hypoxia increased CRF and urocortin 1 but strongly decreased CRF-BP mRNA expression. We show that CRF is expressed in tadpole tail, is up-regulated by environmental stressors, and is cytoprotective. The inhibitory binding protein for CRF is regulated by hormones or by environmental stressors and can modulate CRF bioactivity. (*Endocrinology* 147: 1498–1507, 2006)**

AMPHIBIAN METAMORPHOSIS is controlled by thyroid hormone ($T_3$), which acts on target tissues to induce gene expression programs that effect tissue transformation (1). Gene expression screens have identified $T_3$ target genes in different tadpole tissues (2–5). One of 17 $T_3$-inducible genes isolated in a screen of *Xenopus laevis* tadpole tail (2) was later identified as the corticotropin-releasing factor (CRF) binding protein (CRF-BP) (6).

CRF and related peptides (*i.e.* urocortins) are the principal vertebrate stress neuropeptides, mediating diverse developmental, physiological, behavioral, and autonomic responses to stress (7). These actions are transduced by two G protein-coupled receptors, CRF type 1 ($CRF_1$) and CRF type 2 ($CRF_2$) (8). CRF regulates pituitary secretion in vertebrates (9, 10), but it is also found in hypothalamic and extrahypothalamic brain areas where it functions as a neurotransmitter/neuromodulator (7, 11). CRF and related peptides are expressed in diverse extraneural tissues where they may function as paracrine regulators (12–16). They protect neuronal and cardiac cells from apoptosis (17–21) and induce proliferation in cultured mammalian cells (22–24).

A secreted binding protein for CRF, the CRF-BP was originally isolated and characterized from human liver and rat brain (25). A major function of the CRF-BP is to modulate access of CRF to CRF receptors (26). The dramatic up-regulation of CRF-BP mRNA in tadpole tail during spontaneous metamorphosis or by $T_3$ treatment (6, 27) prompted us to investigate a physiological role for CRF and CRF-BP in the tail. If CRF is expressed in the tadpole tail, we hypothesized that it might act as a cytoprotective or growth factor before metamorphic climax. In this model of CRF action, the up-regulation of CRF-BP at metamorphic climax would limit CRF bioavailability, thus promoting tail resorption. We also hypothesized that up-regulation of CRF or down-regulation of CRF-BP in response to environmental insults could play an adaptive role in maintaining the viability of the tadpole tail before metamorphosis, a critical locomotory structure without which the animal could not feed or escape predation.

To test this model for CRF and CRF-BP actions, we first determined that CRF and CRF receptors are expressed in *X. laevis* tadpole tail and in the tail myoblast-derived cell line XLT-15 (28). We next used tail explant cultures and XLT-15 cells to test for CRF actions. We found that CRF, acting via the $CRF_1$ receptor, is cytoprotective in tadpole tail; CRF slowed tail regression, reduced caspase 3/7 activity, and induced XLT-15 cell proliferation. We also found that CRF-BP blocks CRF actions on tail cells, and overexpression of CRF-BP in tail muscle cells *in vivo* hastens their demise, thus supporting the hypothesis that the up-regulation of CRF-BP at metamorphic climax limits CRF action and thereby promotes tail resorption.

## Materials and Methods

### *Animal husbandry*

*X. laevis* tadpoles were obtained by in-house breeding or were purchased from Xenopus I (Dexter, MI). Developmental stages were as-

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Abbreviations: CRF, Corticotropin-releasing factor; CRF-BP, CRF binding protein; FBS, fetal bovine serum; GFP, green fluorescent protein; LSD, least significant difference; NF, Nieuwkoop and Faber; xCRF, *X. laevis* CRF.

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signed according to Nieuwkoop and Faber (NF; Nieuwkoop and Faber, 1956). All procedures involving animals were conducted in accordance with the guidelines of the University Committee on the Care and Use of Animals of the University of Michigan.

### *RNA extraction and semiquantitative RT-PCR*

We harvested tails from tadpoles at three developmental stages (premetamorphosis, NF stages 52–53; prometamorphosis, NF stages 56–57; metamorphic climax, NF stages 62–63), snap froze and stored at −80 C until RNA isolation. We extracted total RNA from tails or XLT-15 cells (*X. laevis* tail myoblast-derived cell line; 28) using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

We used semiquantitative RT-PCR to analyze gene expression (Table 1). We treated RNAs with deoxyribonuclease I before RT to eliminate genomic DNA, and RNA (1 μg) was reverse transcribed or not using Superscript II RNase H− reverse transcriptase following the manufacturer's instructions (Invitrogen). For PCR, we used HotStar T*aq* DNA polymerase according to the manufacturer's protocol (QIAGEN, Valencia, CA). PCR primer sequences and conditions are given in Table 1. The number of cycles used for PCR represented the midpoint of a linear amplification curve determined empirically for each gene. PCR products were analyzed on 1.2% agarose gels containing ethidium bromide. PCR with nonreverse transcribed controls produced no bands (data not shown). RNA extractions and RT reactions for each developmental stage were conducted on five independent tail samples. Analysis of gene expression in tail fin compared with tail trunk and in XLT-15 cells was conducted on three samples per treatment.

### *CRF RIA*

We acetic acid-extracted tissues of NF stage 58 tadpoles (brain, tail, body skin) and XLT-15 cells before assay and determined CRF content in tissue extracts using a homologous *X. laevis* CRF (xCRF) RIA as described (29). Total protein content of tissue extracts was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL).

### *CRF-BP cross-linking assay*

We used a chemical cross-linking assay as described (27, 30) to analyze CRF-BP in tissue extracts. We determined the protein content of the supernatant using the bicinchoninic acid method (Pierce Chemical) and used 75 μg protein derived from tails of NF stages 50–52 tadpoles treated with or without 100 nM $T_3$ for 48 h in each cross-linking reaction.

### *Production and purification of recombinant mouse CRF-BP*

We produced recombinant mouse CRF-BP in stably transfected AtT-20 cells (see Ref. 30). Earlier, we showed that recombinant mouse CRF-BP binds radiolabeled frog CRF (27). Two hundred milliliters of conditioned medium was concentrated to 40 ml and allowed to bind to a CRF-affinity column (prepared with Affi-Gel-10, Bio-Rad Laboratories, Richmond, CA) overnight at 4 C. The CRF-BP was eluted with 50 mM sodium acetate (pH 4.0), 20% acetonitrile, and neutralized to pH 7.0 with 1 M $NaHCO_3$. Fractions were analyzed by SDS-PAGE and gels silver stained to determine protein purity and protein concentration.

### *cAMP assays*

To determine whether functional CRF-receptors are expressed in tadpole tail, we cultured tail slices *in vitro*, exposed them to CRF or forskolin, and conducted cAMP assays. The production of cAMP is increased in response to CRF activation of $CRF_1$ or $CRF_2$ receptors in many cell types. Hence, measuring intracellular cAMP is the most direct way to assess the actions of CRF peptides in tail cells and to determine the ability of CRF-BP to attenuate CRF receptor activation and signaling in this system (see below). Transverse tail sections (from NF stage 59 tadpoles) were made with a scalpel and placed individually into wells of 12-well plates and cultured in amphibian-strength DMEM (0.66×) supplemented with 2 mM $NaHCO_3$, 0.01% bacitracin, 0.1 mM ascorbic acid, and 1× antibiotic-antimycotic (Invitrogen). Tail slices were cultured at 25 C under a humidified atmosphere of 5% $CO_2$ and 95% $O_2$ with gentle rotation (50 rpm). Tissues were washed and preincubated for 1 h in stimulation medium (1 mM 3-isobutyl-1-methylxanthine in DMEM), then treated with or without 500 nM *Xenopus* CRF or 5 μM forskolin for 2 h. We then processed tissue for cAMP determinations by ELISA following the manufacturer's instructions (GE Healthcare, Piscataway, NJ).

We plated XLT-15 cells into either six-well ($1 \times 10^5$ cells/well) or 12-well ($4 \times 10^4$ cells/well) dishes and cultured them overnight at 25 C under an atmosphere of 5% $CO_2$ in amphibian-strength Leibovitz' medium (0.66×) containing 10% $T_3$-stripped fetal bovine serum (FBS). Cells were preincubated in stimulation medium (L-15 without FBS; 1 mM 3-isobutyl-1-methylxanthine) for 1 h then exposed to peptides or drugs for 10 min. The medium was then aspirated, lysis buffer was added, and supernatants were stored at −20 C until assay for intracellular cAMP concentrations.

To determine whether purified, recombinant mouse CRF-BP alters xCRF-induced cAMP accumulation, we preincubated XLT-15 cells as described above, then added xCRF (20 nM), rmCRF-BP (40 nM), or xCRF plus rmCRF-BP to the cells. The xCRF and rmCRF-BP were combined and incubated for 1 h at 4 C before addition to cells. Cells were incubated for 10 min before harvest and assay for cAMP.

### *In vitro tadpole tail regression assay*

We dissected tails from NF stages 58–59 tadpoles and cultured them in wells of six-well plates (three tails per well; three wells per treatment) in DMEM (0.66×). Treatments were initiated on the day of tissue isolation, and the medium was changed and treatments replenished daily. We captured digital images of tails and measured tail area using Scion image software (version 4.0; Scion Corp., Frederick, MD).

### *[$^3$H]Thymidine uptake assay*

We plated XLT-15 cells in 12-well plates ($2 \times 10^4$ cells/well) and cultured them in L-15 (0.66×) containing 0.5% $T_3$-stripped FBS. We used reduced serum because pilot studies established that 0.5% FBS main-

**TABLE 1.** Primer sequences and reaction conditions used for semiquantitative RT-PCR analysis of gene expression in *X. laevis* tail or XLT-15 cells

| Gene | Primer (forward *above*, reverse *below*; 5′–3′) | PCR product (bp) | Annealing temperature (C) | No. of cycles | GenBank accession no. |
|---|---|---|---|---|---|
| CRF | TCTCCTGCCTGCTCTGTCCAA<br>CTTGCCATTTCTAAGACTTCACGG | 321 | 61 | 37 | S50096 |
| $CRF_1$ receptor | GATCTAAATAGCAGGATGCTGTTGGC<br>GCCGTTCAGGTGACATTCTCTGTAC | 288 | 63 | 40 | Y14036 |
| $CRF_2$ receptor | GTACCTCCCACATCCCACAGCTTCAC<br>GACGCCCAGGTTCCATTCTCAAAGC | 308 | 63 | 40 | Y14037 |
| CRF-BP | TGACTCCTGCTTCCAGACCT<br>TGACCTTGTAATGCTCCCCAC | 452 | 61 | 37 | U41858 |
| Urocortin 1 | GGGTTAAATGGGCTGTTAGGTGATG<br>GGCAATCTCTATCATCTGTCTGAG | 309 | 55 | 35 | AY596827 |
| rpL8 | CACAGAAAGGGTGCTGCTAAG<br>CAGGATGGGTTTGTCAATACG | 477 | 63 | 31 | U00920 |

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tained cell viability and a low rate of cell proliferation (data not shown). This allowed us to analyze effects of treatments on cell proliferation. Treatments included: no additions, 10 nM CRF, and, for comparison, 10% $T_3$-stripped FBS. In separate experiments, we tested the effects of immunoneutralization of endogenous CRF by adding either 2.4 $\mu$g/ml control IgG (purified from nonimmune rabbit serum) or 2.4 $\mu$g/ml anti-xCRF IgG (purified as in 31) to the cultures. For both experiments, we cultured cells for 48 h in treatments, then added [$^3$H]thymidine (0.75 $\mu$Ci/well; PerkinElmer Life and Analytical Sciences, Boston, MA) and continued the incubation for 6 h. Cells were then washed with ice-cold PBS, fixed in 5% trichloroacetic acid, lysed in 1 N NaOH, and radioactivity measured in cell extracts. Each experiment was conducted twice with four replicate wells per treatment to verify results.

We stably expressed xCRF-BP in XLT-15 cells and measured effects on [$^3$H]thymidine uptake. We transfected cells with 1 $\mu$g CMVneo (empty vector) or CMV-xCRF-BP (a xCRF-BP expression vector) (27) using the FuGENE-6 reagent following the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Transfected cells were cultured in L-15 (0.66×) plus 10% $T_3$-stripped FBS for 2 d before initiating selection with G418 (800 $\mu$g/ml). We did not select individual clones; instead, we propagated two pools of cells that were stably transfected with either the empty vector or the xCRF-BP expressing vector. We conducted [$^3$H]thymidine uptake assays as described above.

### *Assay of caspase 3/7 activity in tadpole tail explants*

We dissected tails from late prometamorphic tadpoles (NF stage 58) and cultured them as described above with or without hormones (10 or 100 nM xCRF or 100 nM $T_3$). After 4 d, we harvested tails and determined caspase 3/7 activity using the Caspase 3/7 Glo Assay kit (Promega, Madison, WI) following the manufacturer's instructions with modifications. Tails were homogenized in 0.5 ml PBS (0.05 M sodium phosphate, 0.9% saline, pH 7.4) containing 0.1% Triton X-100. Values are expressed as relative light units per microgram of protein. Two independent experiments were conducted with four to five replicate tails per treatment.

### *Electroporation-mediated gene transfer*

We transfected tail muscle cells of living tadpoles (NF stage 58) by electroporation-mediated gene transfer following methods of Haas *et al.* (32) and Nakajima and Yaoita (33) with modifications. Muscle cells were coelectroporated with pEGFP-N3 (Clontech, Palo Alto, CA) and one of the following: TOPO-xCRF, CMV-xCRF-BP, CMV-mCRF-BP, or CMV-neo (empty vector). The TOPO-xCRF vector was constructed by subcloning the full-length cDNA for the xCRF precursor into pcDNA-TOPO3.1 (Invitrogen). The functionality of this expression vector was confirmed by transfection of PC-12 cells and analysis of CRF peptide by RIA. The construction and validation of the CMV-xCRF-BP expression vector are described by Valverde *et al.* (27) and the CMV-mCRF-BP vector by Cortright *et al.* (30).

For electroporation, we anesthetized tadpoles, backfilled glass micropipettes with DNA solution (0.75 $\mu$g DNA/$\mu$l $H_2O$) plus fast green dye (0.01%) using a Drummond microinjector, and delivered 80 nl in a single injection. A custom-made electrode comprised of two parallel flat platinum plates (2-mm width) was placed over the injection site using a micromanipulator and five square pulses of 30 V (11-msec duration, 0-msec delay) were delivered using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI). The polarity was reversed, and five more pulses were delivered. Green fluorescent protein (GFP) expression was observed by 24 h and increased up to 72 h after electroporation. For image capture, we used an Olympus IX81 inverted fluorescence microscope and MetaMorph software (version 6.1; Universal Imaging Corp., Downingtown, PA) to analyze cell area and GFP intensity.

### *Exposure of tadpole tail explants to environmental stressors*

We dissected tails from NF stage 58 tadpoles and cultured them individually in 30-mm tissue culture dishes (n = 4/treatment) in L-15 (0.66×) without FBS. After overnight culture, we changed the medium and initiated treatments that were continued for 6 h before harvesting tissues for RNA isolation and semiquantitative RT-PCR (see above). Treatments included control [L-15 (0.66)×), 25 C, 5% $CO_2$, humidified atmosphere), hypoxia [L-15 (0.66×), 25 C; dishes placed in tissue culture chamber and atmosphere exchanged with nitrogen], thermal stress [L-15 (0.66×), 33 C, 5% $CO_2$], hypersmotic stress [L-15 (1.33×) = 400 mOsm, 25 C, 5% $CO_2$], and desiccation (culture medium removed, 25 C, 5% $CO_2$).

### *Statistical analyses*

We analyzed data by one- or two-way ANOVA or by Student's unpaired *t* test using the SYSTAT computer program (version 10; SPSS Inc., Chicago, IL). Data were $\log_{10}$ transformed before analysis when variances were found to be heterogeneous. Analysis of derived values (*i.e.* percentages) was done on arc-sin-transformed data. After ANOVA, we used Fisher's least significant difference (LSD) multiple contrast test or Scheffé's test to determine pair-wise differences ($P < 0.05$).

## Results

### *Components of the CRF signaling pathway are expressed in tadpole tail and XLT-15 cells*

*mRNAs.* Using RT-PCR we found CRF and $CRF_1$ mRNAs in tadpole tail at early prometamorphosis, mid-late prometamorphosis, and metamorphic climax; we did not detect $CRF_2$ receptor mRNA (Fig. 1A). CRF-BP mRNA was expressed from early prometamorphosis and increased strongly at metamorphic climax (Fig. 1A). Tail fin or trunk (muscle plus notochord) were dissected from tails of NF stages 59–62 tadpoles. We found CRF and CRF-BP mRNAs in both tail fin and trunk, but levels were higher in the trunk for both genes (Fig. 1B). There was strong expression of $CRF_1$ receptor mRNA in trunk but not in fin (Fig. 1B).

XLT-15 cells expressed CRF and $CRF_1$ receptor mRNAs but not $CRF_2$ or CRF-BP (Fig. 1C), and treatment with $T_3$ had no effect on gene expression.

*Proteins.* We used a specific xCRF RIA to analyze CRF peptide content in tadpole tissues (29). Serial dilutions of tadpole tail (NF stages 58–59) or XLT-15 cell extract produced displacement curves parallel to the xCRF standard (data not shown). In contrast, CRF was nondetectable in extracts from tadpole body skin (outside of tail). Peptide content was similar in tail (0.93 ± 0.42 pg/$\mu$g protein) and XLT-15 cells (0.87 ± 0.24 pg/$\mu$g protein) but was five times lower than in tadpole brain (4.84 ± 0.82 pg/$\mu$g protein; n = 3).

We also detected CRF-BP protein of the appropriate molecular mass (~37 kDa) in tadpole tail (NF stage 52) by cross-linking assay (27, 30). Binding of [$^{125}$I]xCRF was completely displaced by coincubation with radio-inert xCRF (1 $\mu$M; Fig. 1D). Treatment with 100 nM $T_3$ for 48 h increased tail CRF-BP protein (Fig. 1D).

Both tadpole tail and XLT-15 cells express functional $CRF_1$ receptors. Treatment of tail slices with xCRF (500 nM) for 2 h produced a small but significant increase in tissue cAMP content compared with untreated controls (control, 39.04 ± 1.80; xCRF, 45.84 ± 0.59; 5 $\mu$M forskolin, 317.82 ± 58.93; Fisher's LSD; $P < 0.05$; ANOVA, $F_{(2,8)}$=21.818; $P = 0.002$; experiment was repeated with similar results). Forskolin was included as a positive control. The relatively small increase in cAMP induced by CRF could be due to limitations to tissue penetration of the peptide in explant culture or the time point analyzed. It is also possible that CRF signaling in tail is mediated predominantly by G-proteins other than Gs as has

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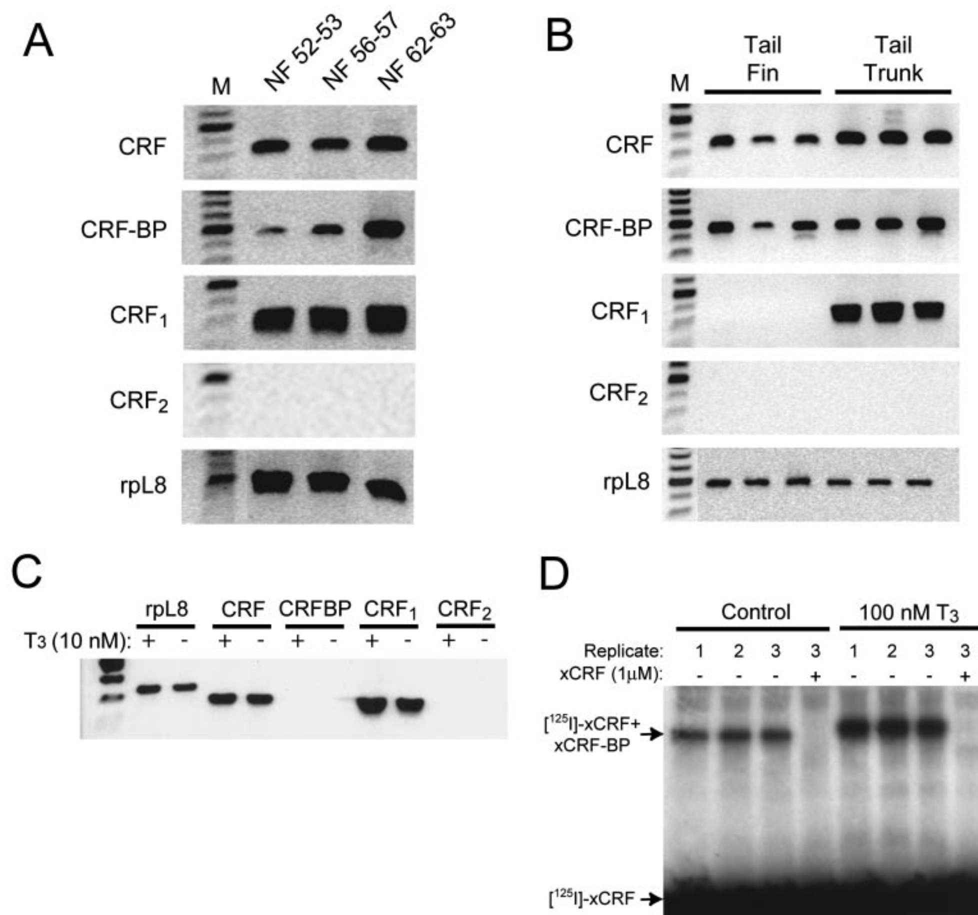


FIG. 1. Expression of CRF, CRF receptor ($CRF_1$ and $CRF_2$), and CRF-BP mRNAs as analyzed by RT-PCR and CRF-BP protein as analyzed by cross-linking assay in *X. laevis* tadpole tail. A, Representative gel (n = 5) of gene expression in tadpole tail throughout metamorphosis. B, Gene expression in tadpole tail fin or tail trunk region. Three independent samples for each tissue region are shown. C, Representative gel (n = 3) of gene expression in XLT-15 cells treated with or without $T_3$ (10 nM) for 48 h. The *X. laevis* ribosomal protein L8 gene (rpL8) was included to control for RNA loading in each experiment. D, Cross-linking assay for CRF-BP using [$^{125}$I]-xCRF and protein (75 μg) extracted from tadpole tails (NF 50–52); tadpoles were treated with or without 50 nM $T_3$ for 48 h before tissue harvest. Shown are three independent samples for each treatment. Specific binding is shown by the complete displacement of [$^{125}$I]-xCRF by the addition of 1 μM radio-inert xCRF before cross-linking.

been shown in some mammalian cells (8). Nevertheless, our results demonstrate functional CRF receptors in tadpole tail.

Treatment of XLT-15 cells with xCRF (100 nM) for 3 min increased intracellular cAMP concentrations by 4-fold (Fig. 2A; $F_{(3,7)}=115.376$; $P < 0.0001$). Intracellular cAMP concentrations continued to increase and at 10 min were 8-fold higher than in unstimulated cells; urocortin 1 produced a similar stimulation (Fig. 2A). The cAMP responses to CRF at 3 min were blocked by coincubation with the general CRF-receptor antagonist αhelicalCRF $_{(9-41)}$ or the $CRF_1$ receptor-specific antagonist antalarmin [Fig. 2B; $F_{(5,12)} = 9.954$; $P = 0.0006$].

### *CRF slows the rate of spontaneous tail regression in vitro*

Treatment of tail explants from NF 58–59 tadpoles with 100 nM xCRF significantly slowed the rate of spontaneous regression (Fig. 3, *upper panel*). Two-way ANOVA showed significant effects of time and treatment, and time by treatment interactions. Separate univariate contrasts with time as the factor showed that treatments diverged at d 4 (Fig. 4A; $F_{(1,8)} = 5.588$; $P = 0.0457$).

A second experiment tested whether the specific $CRF_1$ receptor antagonist antalarmin could block CRF effects on tail regression. Two-way ANOVA showed significant effects of time and treatment and time by treatment interactions. Separate univariate contrasts with time as the factor showed that treatments diverged at d 3 in this experiment [Fig. 3, *lower panel*; $F_{(3,16)} = 4.870$; $P = 0.0136$] incubation of CRF with antalarmin completely blocked the action of CRF on spontaneous tail regression (Fig. 3, *lower panel*).

### *CRF decreases caspase 3/7 activity in tail explant cultures*

We treated tails derived from NF stages 58–59 tadpoles with two doses of CRF (10 or 100 nM) or one dose of $T_3$ (100 nM) for 4 d before harvest and assay for caspase 3/7 activity. The 100 nM dose of CRF produced a statistically significant reduction in caspase 3/7 activity [Fig. 4; $P < 0.05$, Scheffé's test; ANOVA $F_{(3,14)} = 47.535$, $P < 0.0001$]. In contrast, $T_3$ caused an 8- to 10-fold increase in caspase 3/7 activity.

### *CRF increases [$^3$H]thymidine uptake by XLT-15 cells*

Treatment with CRF (10 nM) significantly increased [$^3$H]thymidine uptake by XLT-15 cells [control (0.5% FBS),

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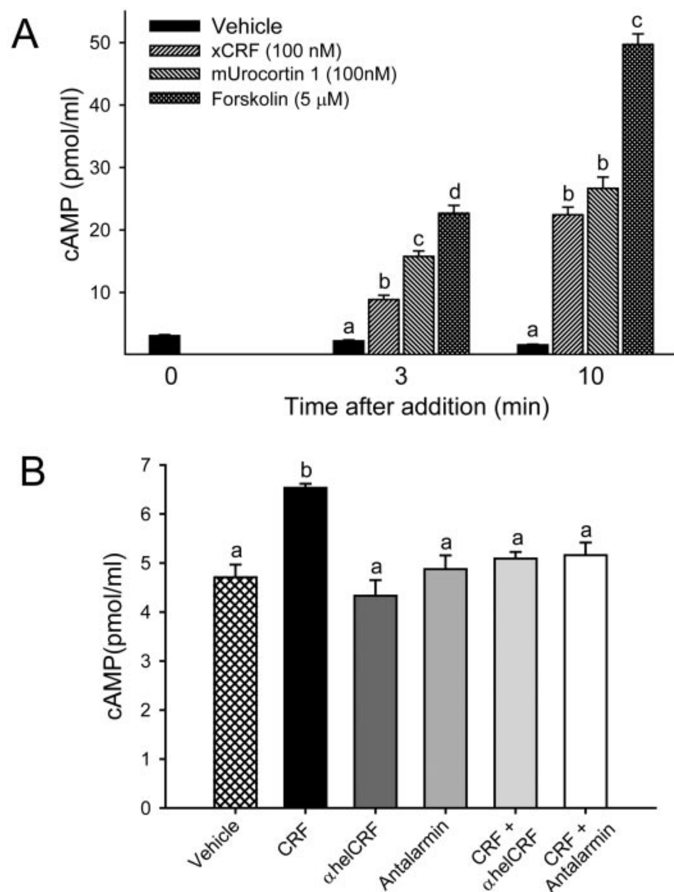


FIG. 2. Stimulation of cAMP in XLT-15 cells by CRF and urocortin and blockade by CRF antagonists. A, XLT-15 cells were treated with vehicle, *Xenopus* CRF (xCRF; 100 nM), mouse urocortin (mUrocortin; 100 nM), or forskolin (5 μM) for 0, 3, or 10 min before cAMP assay. *Bars*, Mean ± SEM (n = 3/treatment). *Letters*, Pair-wise differences among treatment means within a time point (Fisher's LSD; $P < 0.05$). B, XLT-15 cells were exposed to xCRF (100 nM), αhelicalCRF$_{(9-41)}$ (1 μM), antalarmin (1 μM), or xCRF + antalarmin for 3 min before harvest and assay for cAMP. *Bars*, Mean ± SEM (n = 3/treatment). *Letters*, Pair-wise differences among treatment means (Fisher's LSD; $P < 0.05$).

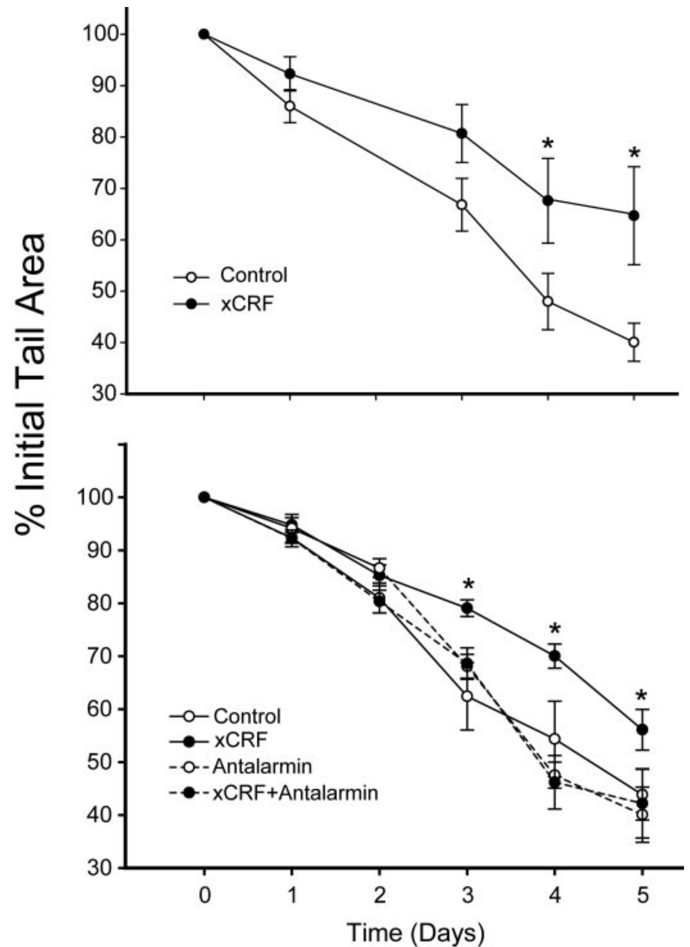


FIG. 3. CRF slows spontaneous tail regression *in vitro*. Tails from NF stages 58–59 tadpoles were cultured for 5 d, and images were digitally captured each day and analyzed for tail area. *Upper panel*, Treatment with CRF (100 nM) slows spontaneous tail regression. *Lower panel*, $CRF_1$ receptor antagonist antalarmin blocks the effect of CRF on tail regression. Tails were treated with 100 nM CRF, 1 μM antalarmin, or CRF + antalarmin. Data for both experiments are expressed as mean percentage of initial tail area ± SEM (n = 3/treatment). *Asterisks*, Significant differences from the control value at a given time point (ANOVA on arc-sin-transformed data; $P < 0.05$).

2392.75 ± 290.67 cpm/well; xCRF (10 nM), 3342.50 ± 222.62 cpm/well; 10% FBS, 5100.50 ± 201.08; n = 4/treatment; Fisher's LSD, $P < 0.05$, ANOVA, $F_{(3,15)} = 71.904$; $P < 0.001$; results representative of two independent experiments].

In separate experiments, we tested whether immunoneutralization of endogenous CRF alters [$^3$H]thymidine uptake. Incubation with affinity-purified IgG against xCRF significantly reduced [$^3$H]thymidine uptake by approximately 50% [control IgG (0.5% FBS), 6750.00 ± 391.92 cpm/well; α-xCRF IgG, 3255.75 ± 539.60; n = 4/treatment; Student's unpaired *t* test; $P < 0.05$; results representative of two independent experiments).

### *CRF-BP blocks the cAMP response to CRF and decreases [$^3$H]thymidine uptake in XLT-15 cells*

We tested whether the addition of purified, recombinant mouse CRF-BP to XLT-15 cells alters cAMP responses to CRF. As before, xCRF significantly increased intracellular cAMP concentration [Fig. 5A; ANOVA, $F_{(3,11)} = 7.434$; $P = 0.011$], but preincubation of xCRF with rmCRF-BP completely blocked CRF action. Addition of rmCRF-BP alone to the cell culture did not significantly alter cAMP accumulation.

Stable overexpression of xCRF-BP in XLT-15 cells reduced [$^3$H]thymidine uptake compared with empty vector controls (Fig. 5B; Student's unpaired *t* test; $P = 0.004$).

### *Effects of overexpression of CRF or CRF-BP in tadpole tail muscle cells in vivo*

We transfected tadpole tail muscle cells *in vivo* with vectors to overexpress either xCRF or CRF-BP and monitored GFP expression over time. Overexpression of xCRF tended to maintain GFP expression (both intensity and cell area), although this effect was not statistically significant. In contrast, overexpression of CRF-BP accelerated the loss of GFP fluorescence, which was statistically significant for the mouse

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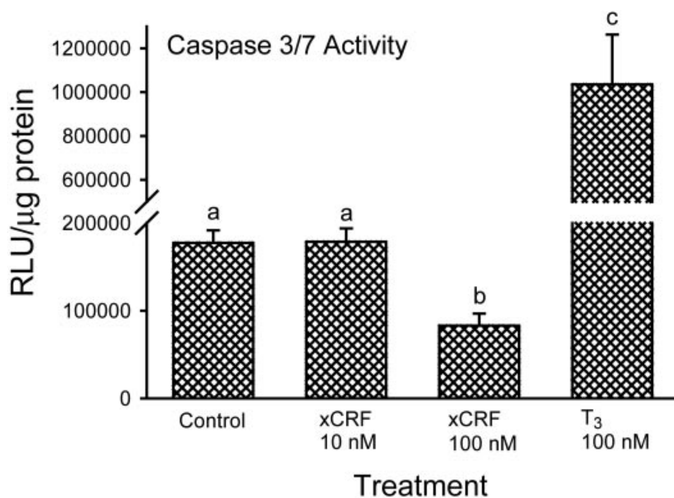


FIG. 4. CRF decreases caspase 3/7 activity in tadpole tail explants. Tails from late prometamorphic tadpoles (NF stage 58) were cultured with or without hormones (10 or 100 nM xCRF or 100 nM $T_3$) for 4 d before harvest and assay of caspase 3/7 activity. Two independent experiments were conducted with four to five replicate tails per treatment. Data from one experiment are shown. *Bars*, Means ± SEM. *Letters* indicate pair-wise differences among treatments (Scheffé's test, $P < 0.05$).

CRF-BP vector [Fig. 5, C and D; Scheffé's test, $P < 0.05$; ANOVA, $F_{(3,23)} = 2.978$, $P = 0.049$; the xCRF-BP treatment approached significance at $P = 0.068$].

*Effects of environmental insults on gene expression in tadpole tail explants*

Exposure of tail explants to hypoxic conditions caused statistically significant increases in both CRF (Student's unpaired *t* test, $P = 0.048$) and urocortin 1 mRNAs ($P = 0.019$) but strongly down-regulated CRF-BP mRNA ($P < 0.001$; n = 4/treatment; Fig. 6). Other treatments tended to up-regulate CRF (except thermal stress) and urocortin 1 mRNAs, and this was statistically significant for desiccation for CRF ($P = 0.032$) and hyperosmotic medium for urocortin 1 ($P = 0.22$). Besides hypoxia, none of the other treatments altered CRF-BP expression, and no treatments affected $CRF_1$ receptor mRNA.

## Discussion

We show a cytoprotective role for CRF in the tadpole tail and the integration of ligand, receptor, and hormone binding protein in the regulation of tail muscle cell survival. Earlier work showed CRF-BP is expressed in the tail and is dramatically up-regulated during metamorphosis (6, 27). Findings in mammalian cells showing cytoprotective and proliferative effects led us to hypothesize that CRF peptides play similar roles in the tadpole tail and that these actions may be neutralized by the CRF-BP, especially at metamorphic climax. We first showed that CRF and CRF receptors are expressed in the tail. We then found that CRF slows spontaneous resorption and decreases caspase 3/7 activity in tail cultures and increases [$^3$H]thymidine uptake in XLT-15 cells. The actions of CRF on tail or XLT-15 cells were blocked by coincubation with the specific $CRF_1$ receptor antagonist antalarmin or by CRF-BP. Furthermore, overexpression of CRF-BP in tail muscle cells *in vivo* hastened their demise. Taken together, our findings provide strong support for an evolutionarily conserved cytoprotective role for CRF peptides that can be neutralized by the up-regulation of the inhibitory CRF-BP *in vivo*.

We found mRNAs and functional proteins for CRF, the $CRF_1$ receptor, and the CRF-BP in tadpole tail throughout metamorphosis. Widespread expression of CRF peptides and receptors at sites outside the central nervous system and pituitary has been found in mammals and in frogs (8, 16). We did not detect the $CRF_2$ receptor in the tail or in XLT-15 cells. This contrasts with widespread expression of this receptor in peripheral tissues of adult mammals and frogs, whereas the $CRF_1$ receptor is expressed predominantly in the brain and pituitary (16, 34–36). Expression of $CRF_1$ and $CRF_2$ mRNAs have been reported in embryonic and fetal tissues in mammals (37, 38); thus, it is likely that both receptors have functions in peripheral tissues during early development, whereas the $CRF_2$ becomes the predominant peripheral receptor in adulthood.

We found that treatment of tadpole tails with CRF *in vitro* slowed the rate of spontaneous resorption. For our studies, we chose late prometamorphic tadpoles because their tails spontaneously regress *in vitro*; the rate of regression is positively correlated with the stage of metamorphosis and thus endogenous plasma $T_3$ titers (9, 39). We used a dose of 100 nM xCRF in our *in vitro* studies, and although we do not know what the local concentration of CRF is in interstitial fluids within the tail, our RIA of tail extracts suggests that the lowest estimate would be in the low nanomolar range (based on tissue weight, but it is likely to be considerably higher). The action of CRF on tail resorption was blocked by coincubation with the specific $CRF_1$ receptor antagonist antalarmin. In mammals, CRF and related peptides are cytoprotective for neuronal and cardiac cells (17–19, 40), and these effects were shown to be mediated by the $CRF_1$ receptor (40). We found a similar dependence on the $CRF_1$ in tadpole tail.

Because CRF significantly reduced caspase 3/7 activity in tail explants, we interpret this action of CRF as cytoprotective. The predominant form of apoptotic cell death entails the activation of a proteolytic cascade involving cysteine proteases called caspases (41). The activity measured in our study (caspase 3 and 7) represents a terminal event in the caspase pathway, and these enzymes are normally activated during tail regression in the tadpole (42). Recent work showed that CRF-mediated cytoprotection in human retinoblastoma cells is linked to the inhibition of caspase-dependent apoptosis (43).

In addition to a cytoprotective action, we found that CRF can induce proliferation of XLT-15 cells as evidenced by increased [$^3$H]thymidine uptake. A role for endogenous CRF in XLT-15 cell proliferation is supported by our findings that XLT-15 cells make CRF and that anti-xCRF IgG (but not control IgG) or the overexpression of CRF-BP decreased [$^3$H]thymidine uptake. Several mammalian cell types proliferate in response to CRF (22, 24). Thus, in addition to protecting tail cells from programmed cell death, CRF pep-

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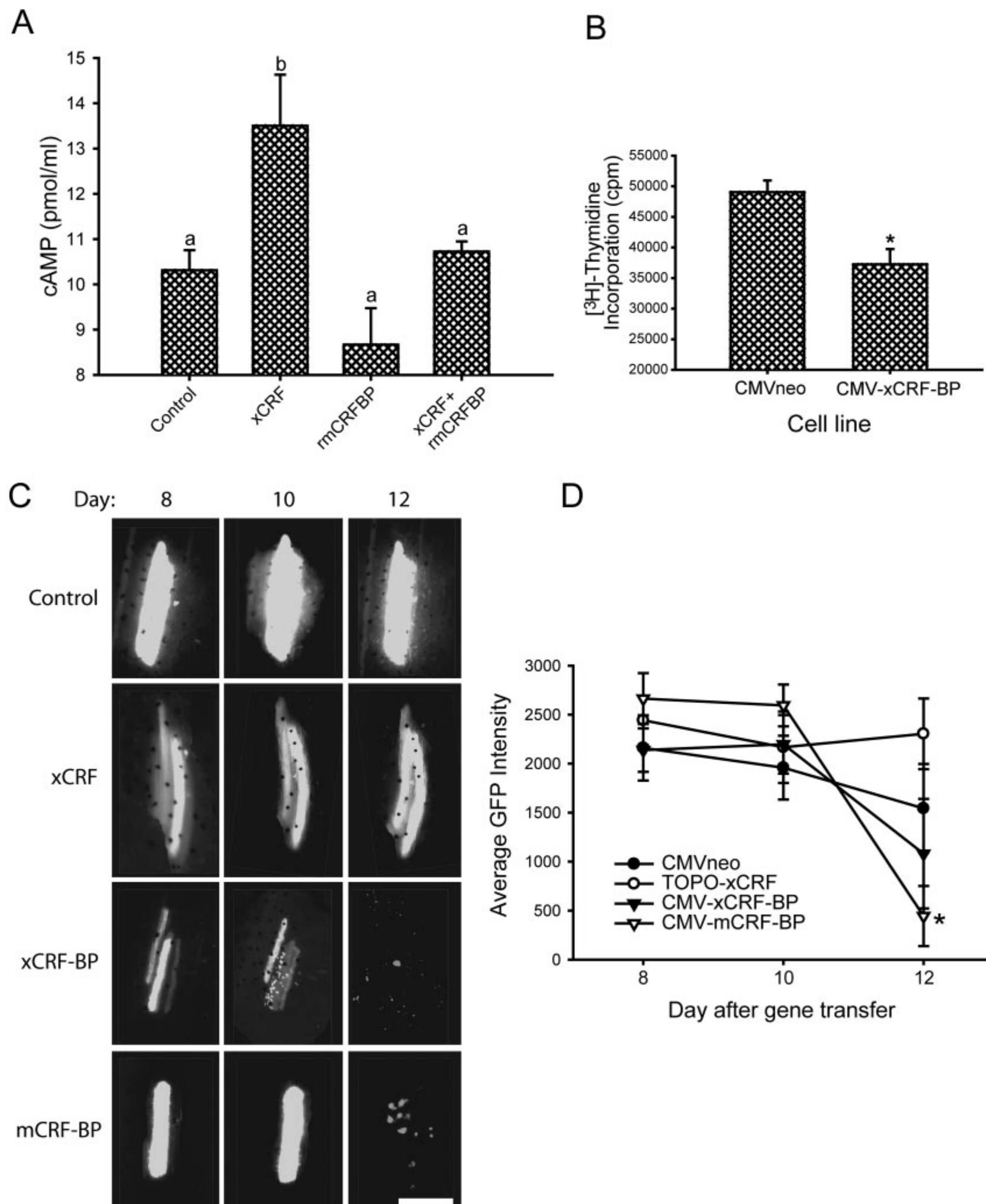


FIG. 5. CRF-BP blocks xCRF-induced cAMP accumulation or [$^3$H]thymidine incorporation in XLT-15 cells, and overexpression of CRF-BP hastens the loss of tail muscle cells *in vivo*. A, Addition of rmCRF-BP inhibits xCRF-induced cAMP accumulation in XLT-15 cells. Cells were treated with 20 nM xCRF, 40 nM rmCRF-BP, or xCRF + rmCRF-BP for 10 min before harvest and assay for cAMP. *Bars*, Mean ± SEM. *Letters*, Significant differences among treatments (Fisher's LSD; $P < 0.05$). B, Overexpression of xCRF-BP reduces [$^3$H]thymidine uptake in XLT-15 cells. Cells were stably transfected with CMV-Neo or CMV-xCRF-BP plasmids. We propagated two pools of cells harboring either of the two plasmids. Stably transfected cells (25,000 cells/well) were cultured in 12-well dishes in L15 (0.66×) with 0.5% $T_3$-stripped serum. After 48 h, 1 μCi [$^3$H]thymidine was added to each well, and cells were then cultured for an additional 48 h before harvest and analysis of [$^3$H]thymidine incorporation. *Bars*, Mean ± SEM (n = 6/treatment). Results are representative of two independent experiments. *Asterisk*, Significant difference from CMV-Neo transfected cells by Student's unpaired *t* test ($P < 0.05$). C, Representative images of GFP expression in tadpole tail muscle cells (NF stage 58) *in vivo* after electroporation-mediated gene transfer. Muscle cells were coelectroporated with pEGFP-N3 and one of the following: TOPO-xCRF, CMV-xCRF-BP, CMV-mCRF-BP, or CMVneo (empty vector). Tadpoles were then reared in aquaria and GFP fluorescence imaged every 2 d thereafter. D, Quantitation of average GFP intensity in electroporated tail muscle cells over the final 4 d of the experiment. *Bars*, Mean ± SEM (n = 6–8/treatment). *Asterisk*, Significant difference from CMV-Neo transfected cells (Scheffé's test, $P < 0.05$).

tides may function to stimulate tail muscle cell proliferation in the premetamorphic tadpole.

Transcripts for the CRF-BP (6, 27) and the protein product (this study) are expressed in tadpole tail and strongly upregulated by $T_3$ causing peak expression at metamorphic climax. In mammals, several lines of evidence suggest that

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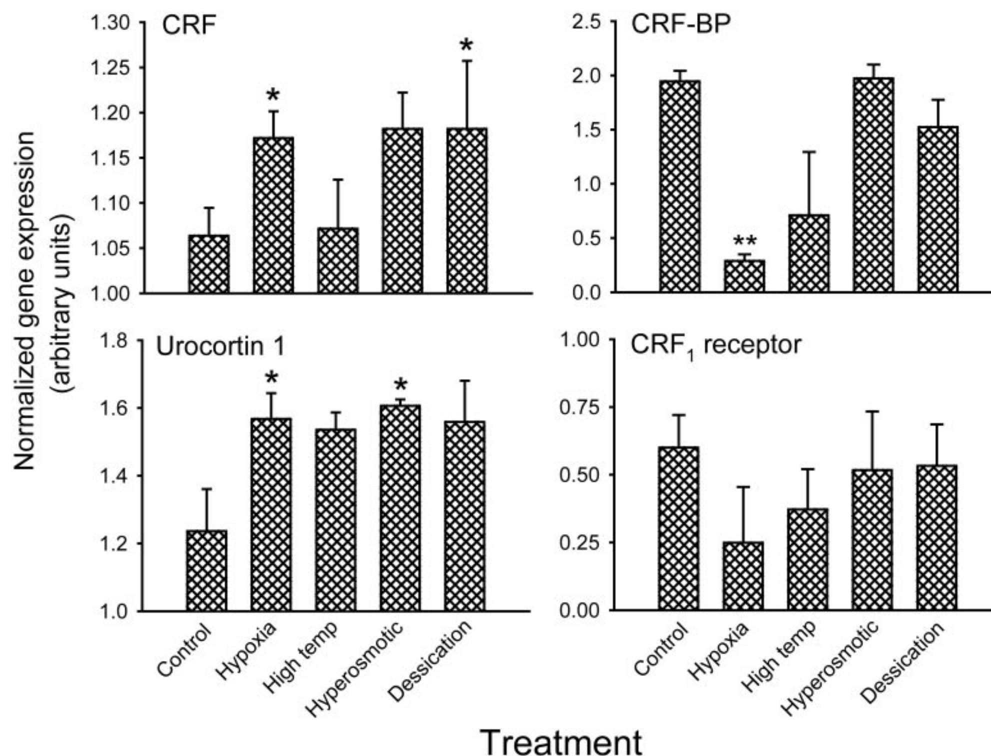


FIG. 6. Effects of different stressors on gene expression in *X. laevis* tadpole tail explants. Tails from NF stage 58 tadpoles were cultured individually in 30-mm tissue culture dishes (n = 4/treatment) in L-15 (0.66×) without FBS and exposed to different stress treatments for 6 h before harvest and analysis of gene expression by semiquantitative RT-PCR. Treatments included control [culture conditions, L-15 (0.66×), 25 C, 5% $CO_2$, humidified atmosphere], hypoxia [culture conditions, L-15 (0.66×), 25 C; dishes placed in tissue culture chamber and atmosphere exchanged with nitrogen], thermal stress [culture conditions, L-15 (0.66×), 33 C, 5% $CO_2$], hypersmotic stress [culture conditions, L-15 (1.33×) = 400 mOsm, 25 C, 5% $CO_2$] and desiccation (culture conditions, medium removed, 25 C, 5% $CO_2$). *Bars*, Mean ± SEM (n = 6/treatment). *Asterisks*, Significant differences from control (Scheffé's test; *, $P < 0.05$; **, $P < 0.001$).

endogenous CRF-BP functions to limit CRF bioavailability (44). We hypothesized that CRF-BP up-regulation at metamorphic climax would serve to bind CRF and thus limit its access to receptors on tail cells. In support of this, we found that addition of rmCRF-BP to XLT-15 cells blocked CRF-dependent increases in intracellular cAMP and that stable overexpression of CRF-BP reduced [$^3$H]thymidine uptake (and the conditioned medium from these transfected cells decreased intracellular cAMP when placed on untransfected XLT-15 cells; data not shown). Finally, using *in vivo* electroporation, we show that overexpression of CRF-BP *in vivo* hastens tail muscle cell loss as evidenced by the disappearance of GFP fluorescence. This effect was observed with two expression vectors (frog or mouse CRF-BP, although statistically significant only with the mouse). Taken together, our findings are consistent with the hypothesis that the up-regulation of CRF-BP at metamorphic climax limits CRF bioavailability, thus promoting tail regression.

Although we have not completed histological localization of the components of the CRF signaling system in tadpole tail, based on several lines of evidence, we can predict where these proteins are expressed. First, our RT-PCR analyses of tail fin (epithelial cells but no muscle or notochord) dissected from the rest of the tail (trunk; includes tail muscle and notochord) show that the tail fin, although it expresses CRF, is not a target for the ligand because it does not express CRF receptors. The expression of CRF and $CRF_1$ receptors in the tail trunk and in the myoblast-derived *Xenopus* cell line XLT-15 suggests that CRF can influence tail muscle cell function via autocrine or paracrine pathways. We found the CRF-BP is expressed in both tail fin and tail trunk, but it was not expressed in XLT-15 cells. Berry *et al.* (45), using *in situ* hybridization histochemistry, also failed to detect CRF-BP mRNA in tail muscle but instead localized expression to the myosepta, connective tissue of the tail fin, notochord sheath, and cells between the muscles (45). Thus, we hypothesize that CRF-BP secreted into the interstices surrounding the muscle cells binds CRF and thus neutralizes the actions of the peptide.

The expression of CRF and urocortins is induced by stress in mammalian and frog brain (7, 31), but stressor-dependent expression in peripheral tissues has received little attention. In rat heart, thermal shock caused an increase in urocortin mRNA that is presumed to be cardioprotective (46). If CRF plays a role in maintaining the viability of the tadpole tail, then we predicted that its expression would be up-regulated by exposure to environmental insults that could directly and perhaps negatively impact tail cell survival. The types of stressors that a tadpole experiences in its natural habitat include thermal stress, osmotic stress (both hyper- and hyposmotic), hypoxia, hypercapnia, tissue damage caused by predatory attacks, among others (47). On the other hand, if

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the CRF-BP limits CRF bioavailability, then we predicted that it would be down-regulated by environmental stress. In support of these predictions, we found increased CRF and urocortin 1 mRNAs in tail explants after exposure to different stressors. The most consistent effect (across genes) was hypoxia, which up-regulated CRF and urocortin 1 but strongly down-regulated CRF-BP.

Taken together, our results support the hypothesis that CRF peptides expressed in premetamorphic tadpole tail play a cytoprotective (and perhaps a cell proliferative) role, acting upstream of caspases. The increase in CRF-BP induced by rising plasma $T_3$ titers at metamorphic climax serves to neutralize CRF bioactivity, thus promoting tail regression. Up-regulation of CRF peptides and down-regulation of the CRF-BP by environmental stressors shows that the production and bioavailability of these peptides can be modulated by direct environmental effects on the tail. This leads to the prediction that CRF peptides protect tail cells from death caused by environmental insults, thus maintaining the viability of this essential locomotory organ until metamorphosis.

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