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Structural and Functional Conservation of Vertebrate Corticotropin-Releasing Factor Genes: Evidence for a Critical Role for a Conserved Cyclic AMP Response Element

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Corticotropin-releasing factor (CRF) plays a central role in neuroendocrine, autonomic, immune, and behavioral responses to stressors. We analyzed the proximal promoters of two *Xenopus laevis* CRF genes and found them to be remarkably conserved with mammalian CRF genes. We found several conserved *cis* elements in the frog CRF genes including a cAMP response element (CRE), activator protein 1 binding sites, and glucocorticoid response elements. Exposure to a physical stressor caused a rapid elevation in phosphorylated CRE binding protein (CREB; 20 min) and CRF (1 h) in the anterior preoptic area of juvenile frogs. CREB bound to the putative frog CREs *in vitro*, which was disrupted by point mutations introduced into the CRE. The frog proximal CRF promoters supported basal transcription in transfection assays, and forskolin caused robust transcriptional activation. Mutagenesis of the CRE or overexpression of a dominant-negative CREB reduced forskolin-induced promoter activation. Using electroporation-mediated gene transfer in tadpole brain, we show that the proximal CRF promoters support cAMP or stressor-dependent transcription *in vivo*, which was abolished by mutation of the CRE. Using chromatin immunoprecipitation, we found that CREB associated with the proximal frog CRF promoter *in vivo* in a stressor-dependent manner. These data provide strong support for the hypothesis that stressor-induced CRF gene activation *in vivo* depends on CREB binding to the CRE in the promoter. Our findings show that the basic regulatory elements of the CRF gene responsible for stressor-induced activation arose early in vertebrate evolution and have been maintained by strong positive selection. (*Endocrinology* 148: 2518–2531, 2007)

IN VERTEBRATES, EXPOSURE to physical or emotional stressors leads to the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The central regulator of the HPA axis is corticotropin-releasing factor (CRF), which acts on the pituitary to stimulate ACTH secretion leading to increases in plasma glucocorticoids that influence a wide array of physiological, immune, and behavioral responses. Besides the hypophysiotropic function of hypothalamic CRF, the peptide is widely expressed in the brain in which it functions as a neurotransmitter/neuromodulator influencing stress-related behaviors and sympathetic output (1, 2).

Transcription of the CRF gene in mammals is rapidly induced after exposure to physical stressors, and most current knowledge of CRF gene regulation comes from studies in rodents. The gene structure and nucleotide sequences of the coding and upstream promoter regions are highly conserved among mammalian CRF genes. All consist of two exons and a single intron of approximately 400–800 bp (human 801 bp, rat 687 bp, ovine 745 bp). Although the protein coding sequences of mammalian CRF genes are very similar (~90% sequence similarity among human, rat, and ovine), the highest degree of sequence similarity was found among the 330-bp-long proximal segment of the 5′ flanking region [94% similarity between human and ovine (3); also 94% similarity between human and rat and 90% similarity between ovine and rat (Yao, M., unpublished data)], suggesting its functional importance in transcriptional regulation. This segment contains a number of putative transcription factor binding sites highly conserved among mammalian CRF genes. Using cell transfection assays, it was shown that activity of the human CRF gene can be influenced by protein kinase A [acting via the cAMP-response element (CRE)], protein kinase C [acting via the activator protein 1 (AP1) sites], and glucocorticoid receptor [acting via the glucocorticoid response element (GRE)] pathways (4–14; also see Ref. 15 for review). *In vivo* studies in rodents showed a correlation of increased phosphorylated CRE-binding protein (pCREB) with CRF gene activation in several brain regions [as measured by heteronuclear RNA (hnRNA) expression (16–19)]. Similar results were found with the AP1 protein c-Fos, al-

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Abbreviations: ACREB, Dominant-negative mutant form of CREB; AP1, activator protein 1; ChIP, chromatin immunoprecipitation; CRE, cAMP-response element; CREB, CRE binding protein; CRF, corticotropin-releasing factor; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; EM, electroporation-mediated; GRE, glucocorticoid response element; hnRNA, heteronuclear RNA; HPA, hypothalamic-pituitary-adrenal; IBMX, 3-isobutyl-1-methylxanthine; i.c.v., intracerebroventricular; ir, immunoreactive; LSD, least significant difference; NGFI-B, nerve growth factor induced gene B; NRS, normal rabbit serum; oCRF, ovine CRF; pCREB, phosphorylated CRE-binding protein; POA, preoptic area; RT-qPCR, RT-PCR, and RT-quantitative PCR; rxCREB, recombinant *X. laevis* CREB; SDS, sodium dodecyl sulfate.

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though the kinetics of activation of c-Fos (as measured by mRNA) were delayed, compared with pCREB. This has led some to question whether c-Fos is involved in the rapid stressor-induced activation of the CRF gene (16, 17). Although these studies suggest that these transcription factor binding sites are important for CRF transcription, direct evidence for a role for these *cis* elements in stressor-induced transcriptional activation *in vivo* is lacking.

Limited comparative studies of CRF gene expression in nonmammalian species suggest that the gene regulatory elements responsible for tissue-specific and stressor-dependent gene activation may have arisen early and have been conserved through vertebrate evolution. Some of the most detailed structural and functional analyses of the CRF signaling pathway in a nonmammalian species have been conducted in the South African clawed frog *Xenopus laevis*. *X. laevis* possesses two CRF genes, designated xCRFa and xCRFb, owing to its pseudotetraploid genome (20). The two genes code for mature peptides with identical amino acid sequences. We showed previously that the distribution of CRF neurons in the frog brain is highly conserved when compared with mammals (21). Furthermore, we showed that CRF neurons are strongly activated in discrete brain regions in the frog brain in response to an acute physical stressor (21). These regions included the parvocellular neurons of the anterior preoptic area (POA; a region homologous to the mammalian paraventricular nucleus), medial amygdala, and bed nucleus of the stria terminalis; CRF neurons in each of these brain regions are similarly activated by exposure to physical stressors in mammals (22–33).

In the present study, we analyzed the gene structures of two CRF genes of *X. laevis* and compared the regulatory regions of the frog genes with mammalian CRF genes. We found that the gene organization and sequences of the regulatory regions of the frog CRF genes are highly conserved with mammals. Sequence analysis revealed conserved binding sites for CRE-binding protein (CREB), AP1, glucocorticoid receptor, and nerve growth factor induced gene B (NGFI-B) in the proximal promoters of the frog genes. Other, as-yet-uncharacterized regions of strong sequence similarity among frog and mammal CRF genes suggest an evolutionarily conserved role in gene regulation. We showed that CREB was rapidly phosphorylated in the frog POA after exposure to a shaking/handling stressor, which preceded an increase in CRF immunoreactivity in the same cell populations. We tested the functionality of the putative CREs present in the frog CRF gene promoters using both *in vitro* and *in vivo* approaches. Our data show that this element supports activation of the promoter by the cAMP pathway and is specifically bound by CREB protein *in vitro*. Furthermore, we found that CREB associates with the proximal promoters of the frog CRF genes, and the CRE is required for stressor-dependent gene activation *in vivo*.

Materials and Methods

Animal husbandry

X. laevis tadpoles and juveniles were obtained by in-house breeding or were purchased from Xenopus I (Dexter, MI). Tadpoles were raised in dechlorinated tap water (20–22 C; 12-h light, 12-h dark cycle) and fed Frog Brittle (Nasco, Fort Atkinson, WI) *ad libitum*. Developmental stages were assigned according to Nieuwkoop and Faber (34). For electroporation-mediated gene transfer (see below) tadpoles were anesthetized in 0.002% benzocaine. 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 isolation, RT-PCR, and RT-quantitative PCR (RT-qPCR) analysis

We used RT-PCR to determine whether both CRF genes are expressed in *X. laevis* brain. Total RNA was isolated from the tadpole brain using the TRIZOL Reagent (Invitrogen Corp., Carlsbad, CA). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and random hexamers following the manufacturer's instructions. We designed gene-specific PCR primers that spanned the predicted intronic regions of both frog CRF genes (see supplemental Table 1 for primer sequences, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org). The RT-PCR products were isolated by agarose gel electrophoresis and their sequences determined by direct DNA sequencing.

We also used RT-qPCR to determine the levels of expression of both CRF genes in the brain or the POA/hypothalamic region of unstressed and stressed frogs (see below). We designed gene-specific Taqman primer/probe sets for detecting the primary transcripts (hnRNA) and mRNA of both frog CRF genes (see supplemental Table 2 for primer and probe sequences). Reactions were run using the Fast 7500 real-time PCR system (Applied Biosystems, Foster City, CA). For analysis of expression of CRF hnRNAs and mRNA in unstressed and stressed animals, we used a relative quantitation method and expressed the concentrations of the RT-qPCR products in arbitrary units. For quantification and comparison of expression levels of the two frog CRF genes, we generated standard curves using known concentrations of plasmid DNA containing each of the frog CRF genomic clones.

Sequencing of X. laevis genomic clones and comparative genomic analysis

We designed primers based on the published sequences of the coding regions of the two *X. laevis* CRF genes (20) to sequence their upstream regions using the isolated genomic clones as templates. DNA sequence alignment analysis was conducted using AlignX of Vector NTI Suite 5.5 software (Invitrogen Corp., Carlsbad, CA). The promoter sequences of human (AC090195), chimp (LOC464215), sheep (M22853), mouse (AC141209), and rat (M54987) CRF genes were obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Transcription factor binding sites within the proximal promoters of the CRF genes were predicted using the on-line program Match using a library of mononucleotide weight matrices from TRANSFAC 6.0 (www.gene-regulation.com) with both core similarity and matrix similarity of the transcription factor binding sites set higher than 0.85.

Shaking/handling stressor

The shaking/handling stressor paradigm that we used was described previously (21, 35). Briefly, for immunohistochemical analysis, three juvenile frogs (body weight 5–8 g) were placed into 32-oz white polypropylene containers with 250 ml water. The containers were placed on an orbital shaker and shaken continuously at 100 rpm for various times. Controls were left undisturbed in holding tanks until the animals were killed. The frogs were rapidly killed by submersion in 0.05% benzocaine and tissues collected. Frogs were killed at 0 min, 20 min, 1 h, and 2 h after the initiation of the stressor (n = 3/time point), and the heads were fixed in cold 4% paraformaldehyde. Brains were dissected, postfixed, and submerged in 30% sucrose before snap freezing and transverse cryosectioning at 12 μm.

Immunohistochemistry and morphometric analysis

We used immunohistochemistry to analyze CRF immunoreactive (ir) and pCREB-ir neurons in the frog brain as described previously (21). The rabbit polyclonal antiserum to pCREB that we used was raised against an epitope of mouse CREB that is identical with the sequence of *Xenopus*

CREB (catalog no. 06–519; Upstate Biochemicals, Lake Placid, NY). We used a highly specific affinity-purified anti-xCRF IgG described previously (21). Single immunohistochemistry for pCREB-ir and CRF-ir were conducted using the Vectastain elite ABC (rabbit) and Vector VIP kits (both from Vector Laboratories, Inc., Burlingame, CA) following the manufacturer's protocols (antibodies: 1:500 dilution of rabbit polyclonal anti-phospho-CREB; 15–20 ng/μl of affinity purified polyclonal rabbit IgG raised against synthetic frog CRF; see Ref. 21). We used double-labeling fluorescence immunohistochemistry to determine colocalization of pCREB-ir and CRF-ir following a method that we used previously to colocalize CRF and c-Fos (21). Double-labeling experiments were analyzed by confocal microscopy (using a laser scanning confocal microscope; Zeiss, New York, NY) with optical sections of 1 μm thickness captured through the Z-axis.

We quantified CRF-ir and pCREB-ir in discrete brain regions using MetaMorph software (version 6.2r4). For each antibody, we processed all samples simultaneously under identical conditions. Three sections that contained the anterior preoptic regions were analyzed for each animal. All sections were carefully matched for anatomical level, and digital images were captured at $\times$200 magnification for morphometric analysis. Image analysis was conducted in a blinded manner. The brain regions were isolated using a handmade frame that covered the area of interest. For quantification of pCREB-ir (exclusively nuclear staining), the total number of positive nuclei in the areas of interest were counted automatically, and averages of the three sections were calculated for each animal. For quantification of CRF-ir, the total area of the positive staining particles above a standard density threshold in the selected area was counted automatically, and the mean density for each animal was calculated as the total positive staining area on the multiple sections, divided by the total selected area (see Ref. 21). The effects of the shaking/handling stressor on the numbers of pCREB-ir-positive cells and CRF-ir mean density in the brain regions studied were analyzed by Student's unpaired *t* test ($P < 0.05$) using the SPSS statistical package (version 11.5 for Windows; SPSS Inc., Chicago, IL).

Plasmid constructs

We constructed a *X. laevis* CREB expression vector by isolating a cDNA for the entire coding region of the frog CREB gene by RT-PCR using total RNA isolated from juvenile frog brain. The primers used to amplify the *X. laevis* CREB were (*Hin*dIII and *Bam*HI sites are shown in *lowercase letters*): 5′-cccaagcttGTGTTACATGGTGGGGAAG-3′ and 5′-cgcggatccGCCTCCTAATCAGATTTGTGG-3′. We directionally cloned the frog CREB (xCREB) cDNA into the pSP64 Poly(A) vector (Promega Corp., Madison, WI) to produce pSP64A-xCREB. The orientation and sequence of the construct was confirmed by direct DNA sequencing.

All plasmid reporter constructs were generated using the parent plasmid pGL3-basic (Promega), which contains a modified coding region for firefly luciferase and lacks eukaryotic promoter and enhancer sequences. The numbering system used to describe the frog gene promoter fragments for analysis is based on the translation start sites ATG (+1); the transcription start sites are estimates based on sequence alignment with mammalian CRF genes (Fig. 1). We chose the ATGs as the basis for our numbering because many genes use alternative transcription start sites, and the transcription start sites of the frog CRF genes have not yet been determined. We generated the xCRF promoter constructs by first PCR amplifying DNA fragments using the xCRF genomic clones as templates (20) and oligonucleotide primers harboring *Kpn*I (5′) and *Hin*dIII (3′) restriction sites for directional cloning. The purified PCR products were double digested with *Kpn*I and *Hin*dIII (Promega) and ligated into pGL3-basic in a forward orientation to produce pGL3-xCRFa533 and pGL3-xCRFb576. The pGL3-xCRFa533 contains 533 bp of a xCRFa genomic fragment (−1327 to −795), including 396 bp of 5′ flanking sequence and 137 bp of untranslated region. The pGL3-xCRFb576 contains 576 bp of a xCRFb genomic fragment (−1079 to −504), including 452 bp of 5′ flanking sequence and 124 bp of untranslated region. Although the 5′ and 3′ limits of the two promoter fragments overlap, the sizes of the fragments are not identical due to deletions and insertions in the duplicated genes. We conducted oligonucleotide-directed mutagenesis of the CREs present in the promoter constructs using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sequences of all constructs were confirmed by direct DNA sequencing.

Cell culture and transient transfection

Monolayer cultures of PC-12 cells were maintained in high-glucose DMEM (Sigma, St. Louis, MO) supplemented with 5% bovine calf serum, 5% equine serum (both from HyClone, UT), and antibiotics in a humidified atmosphere of 5% CO_2 at 37 C. Monolayer cultures of a *X. laevis* tail myoblast cell line (XLT-15; these cells express CRF) (36, 37) were maintained in Leibovitz's L15 medium (Invitrogen; diluted 1:1.5 for amphibian cells) supplemented with 10% thyroid hormone-stripped fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and antibiotics and cultured under a humidified atmosphere of 5% CO2 at 25 C. Thyroid hormone removal from fetal bovine serum was conducted following the protocol of Samuels *et al.* (38).

Transient transfections were conducted in triplicate in 24-well plates (Falcon, MA). The pRL-null plasmid (Promega), which contains a promoterless *Renilla* luciferase gene, was cotransfected as an internal control for transfection efficiency. PC-12 cells were plated at a density of 4×10^5 cells/well and XLT-15 cells at a density of 6×10^4 cells/well in 24-well plates 24 h before transfection. For PC-12 cells we conducted transfections using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Each well received 800 ng of reporter plasmid plus 10 ng of pRL-null plasmid (Promega). For transfection of XLT-15 cells, we used FuGene 6 transfection reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. Each well received 100 ng of reporter plasmid plus 2 ng of pRL-null. Twenty-four hours after transfection, the medium was changed to fresh medium with or without 25 μM forskolin (Sigma stock was dissolved in dimethylsulfoxide and added to the medium at 1:1000 dilution). The control group received the same concentration of dimethylsulfoxide as the forskolin-treated group. We found that XLT-15 cells, unlike PC-12 cells, required pretreatment and simultaneous treatment with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (500 nM for 1 h pretreatment before the addition of forskolin) to observe an increase in promoter activity, suggesting that the frog cells express higher phosphodiesterase activity, *e.g.* see Ref. 13.

Cells were harvested at different times, and luciferase activity was measured using a dual-luciferase reporter assay system (Promega). All cell transfection experiments were conducted with three replicate wells per treatment, and each experiment was repeated at least three times. The firefly luciferase activities were normalized to the *Renilla* luciferase activities in each cell lysate and the values are expressed as relative luciferase activity.

EMSA

We conducted EMSA using *in vitro* synthesized *X. laevis* cAMP binding protein (xCREB) or nuclear extracts of juvenile frog brain. We produced recombinant xCREB protein by coupled *in vitro* transcription-translation with the pSP64A-xCREB vector following the manufacturer's instructions (TNT system; Promega).

For nuclear extracts we microdissected the preoptic area/diencephalon region from the brains of juvenile frogs, homogenized the tissues in a hypotonic buffer [10 mM HEPES, 10 mM KCl, 1 mM dithiothreitol (DTT), and protease inhibitors], incubated on ice for 30 min, and then pelleted by centrifugation at 300 $\times$ *g* for 10 min. After homogenization, 0.1 total volume of sucrose restore buffer (containing 6.75% sucrose, 50 mM HEPES, 10 mM KCl, 1 mM DTT, and protease inhibitors) was added, followed by centrifugation. We resuspended the pellet in a nuclear extraction buffer (containing 50 mM HEPES, 10 mM KCl, 1 mM DTT, and protease inhibitors) and incubated it on ice for 40 min with vortexing every 10 min. After centrifugation at 100,000 $\times$ *g* for 1 h at 4 C, the nuclear extract (supernatant) was removed and stored at −80 C. We determined the total protein concentrations of each sample using a standard protein assay (BCA protein assay kit; Pierce Biotechnology, Rockford, IL).

DNA probes were prepared for EMSA by annealing complementary oligonucleotides. End labeling was conducted using the large Klenow fragment of DNA polymerase (Promega) and ^{32}P-labeled dCTP (PerkinElmer, Boston, MA), and the products were purified over Sephadex G50 columns. Sequences of the oligonucleotides are shown in Table 1.

EMSAs were conducted following the methods of Dignam *et al.* (39) with minor modifications. *In vitro*-produced proteins (1 μl of 50 μl TNT reaction) or 1 μl (0.8 μg total protein/μl) of brain nuclear extract were

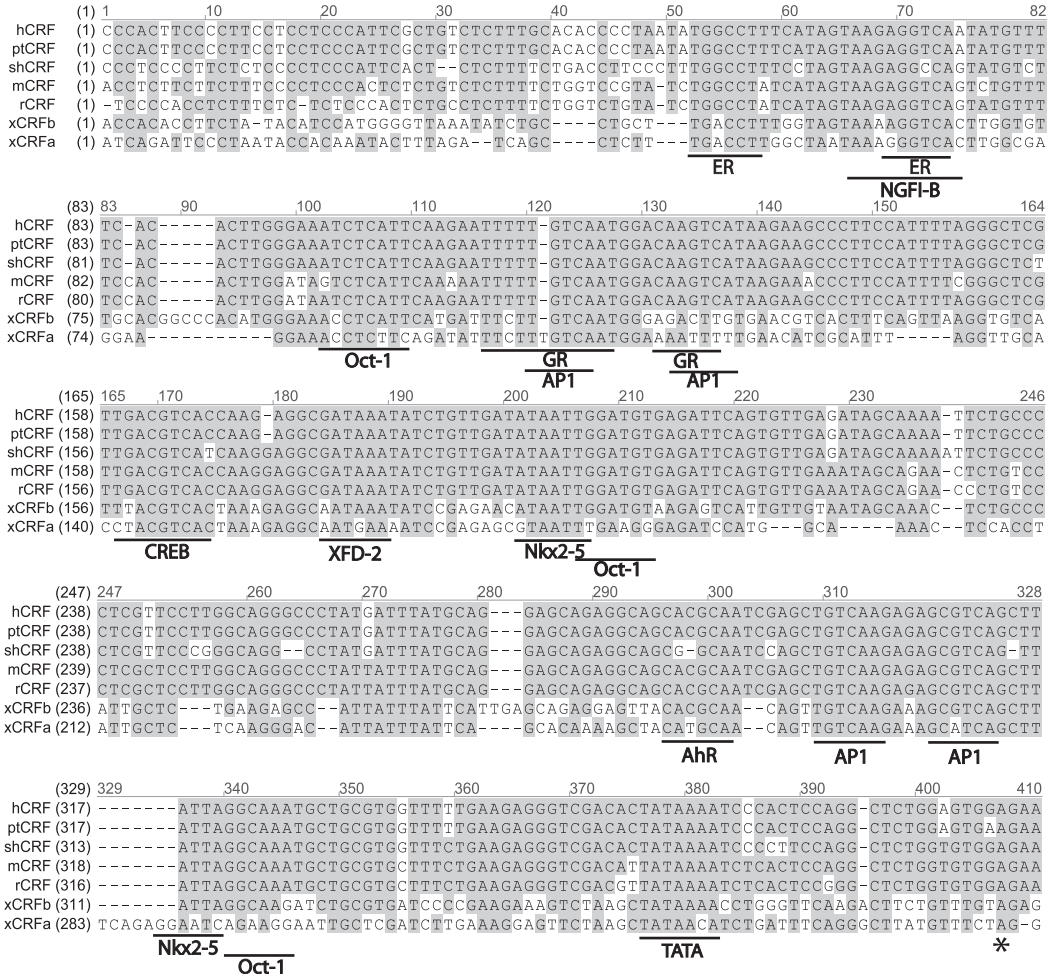

FIG. 1. ClustalW alignment of 5′ promoter regions of human (h), chimp (pt), sheep (sh), mouse (m), rat (r), and *Xenopus* CRF (x) genes. Identical sequences among the CRF genes are *shadowed*. The *asterisk* indicates transcription start sites. Putative binding sites for transcription factors predicted by the on-line program Match using a library of mononucleotide weight matrices from TRANSFAC 6.0 (www.gene-regulation.com) are indicated. ER, Estrogen receptor; Oct-1, octamer binding transcription factor 1; GR, glucocorticoid receptor; XFD2, *Xenopus* forkhead domain factor 2; Nkx 2–5, cardiac-specific homobox protein; AhR, aryl hydrocarbon receptor; TATA, TATA box.

incubated with 20,000 cpm double-stranded ^{32}P-labeled double-stranded oligonucleotides and 1.4 μg double-stranded poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.8), 1 mM DTT, 0.1% IGEPAL CA-630, 50 mM KCl, and 20% glycerol. The reaction continued at room temperature for 40 min before fractionation by 6% nondenaturing PAGE in 0.25× Tris-borate EDTA buffer. The gel was dried and analyzed by autoradiography. Competition was conducted by addition of 100 nM of unlabeled specific or nonspecific oligonucleotides (~100-fold excess) during the incubation. To identify nuclear proteins present in supershifted complexes, we added varying amounts of rabbit anti-CREB serum (Rockland Immunochemicals Inc., Gilbertsville, PA), anti-phospho-CREB serum (catalog no. 06-519, Upstate), or normal rabbit serum (NRS) to the EMSA reaction immediately before adding the oligonucleotide probe.

TABLE 1. Sequences of oligonucleotide probes used in EMSA

DNA probe	Sequence
hCRFCRE	GGCTCGTTGACGTCACCAAGAGGC
xCRFaCRE	GTTGCACCTACGTCACTAAAGAGGC
xCRFbCRE	GTGTCATTTACGTCACTAAAGAGGC
xCRFb[CREmut]	GTGTCATTTA**A**GTC**T**CTAAAGAGGC
xCRFb[CREdel]	GTGTCAT**AGCTAGCT**CTAAAGAGGC

Only upper strands are shown (5′ → 3′). The CRE octomers or the corresponding sequences in the mutated forms are *underlined*, and the mutated bases are shown in *bold*.

Chromatin immunoprecipitation (ChIP) assay

We prepared chromatin from juvenile frog brains and conducted ChIP assay as described by Sachs and Shi (40). For each replicate chromatin preparation, we pooled brain sections containing the POA plus hypothalamus from 10 juvenile frogs (body weight 1–2 g; n = 3–4/treatment). We homogenized the tissue in nuclear extraction buffer (0.5% Triton X-100, 10 mM Tris-HCl, 3 mM $CaCl_2$, 0.25 M sucrose, 1 mM DTT, and protease inhibitor cocktail) and incubated in 37% formaldehyde for 15 min. Nuclei were then pelleted and resuspended in nuclear extraction buffer, followed by homogenization and filtration through a 100-μm cell strainer (BD Biosciences, Bedford, MA). After centrifugation, we resuspended the pellet in sodium dodecyl sulfate (SDS) lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)] and sonicated with five 10-sec pulses at an intensity of five using a Fisherbrand Sonic Dimembrator 100 and pelleted at 20,000 × *g* for 10 min. We then measured the DNA concentration in the supernatant by spectrophotometry, diluted to 100 ng/μl with SDS lysis buffer, and stored the chromatin samples frozen at −80 C until assay.

We conducted ChIP assays with 15 μg chromatin using the Upstate Biochemicals ChIP assay kit following the manufacturer's instructions. After preclearing with protein A agarose/salmon sperm DNA slurry, we

incubated chromatin with primary antibodies overnight at 4 C. We used 5 μl of anti-CREB (Rockland Immunochemicals; based on Ref. 41), anti-acetyl-histone H4 (catalog no. 06–598; Upstate) or NRS in the immunoprecipitations. Immune complexes were precipitated with protein A agarose/salmon sperm DNA, washed, and reverse cross-linked in 0.2 M NaCl overnight at 65 C. After incubation in 40 μg/ml Proteinase K [in 10 mM EDTA, 40 mM Tris-HCl (pH 7.4)] for 1 h at 45 C the DNA was purified using the High Pure PCR product purification kit (Roche) and analyzed by qPCR.

qPCR

For qPCR analysis of ChIP assays, we developed Taqman assays to target the xCRFb core promoter containing the CRE and the coding region (CDS) of CRF genes (the assay recognized both genes and served as a negative control in the ChIP assay; see supplemental Table 3 for primer and probe sequences). Reactions were run using the Fast 7500 real-time PCR system (Applied Biosystems). We applied a relative quantitation method using pooled chromatin as the standard for each primer/probe set. ChIP values for anti-CREB or anti-acetyl-histone H4 were normalized first to the respective input, followed by subtraction of the background signal for ChIP with NRS for each sample.

Electroporation-mediated gene transfer

To test the functionality of promoter elements *in vivo*, we conducted bulk electroporation-mediated gene transfer into tadpole brain as described by Haas *et al.* (42) with minor modifications. *X. laevis* tadpoles (Nieuwkoop and Faber stage 49–51) were anesthetized by immersion in 0.002% benzocaine before intracerebroventricular (i.c.v.) microinjection of 92 nl DNA solution. Each DNA solution contained 1 μg/μl of the reporter plasmid, 50 ng/μl of pRL-null plasmid, 400 ng/μl of pEGFP-N1 plasmid (CLONTECH, Palo Alto, CA; to monitor transfection efficiency), and 0.02% fastgreen dye. Immediately after the injection, we placed a pair of platinum electrodes over the skull and delivered five pulses of 30 V. We reversed the polarity and repeated the current delivery. Animals were allowed to recover and screened for high enhanced green fluorescent protein (EGFP) expression 5 d after the procedure using a MZFLIII fluorescent stereomicroscope (Leica, Bannockburn, IL).

To test the functionality and stress responsiveness of the frog CRF promoters *in vivo*, we administered i.c.v. injections of ovine CRF (oCRF; 20 ng/g body weight) or saline vehicle and waited 6 h before harvesting brains. In a second experiment, we exposed juvenile frogs to shaking/handling stressor as described above. Animals were killed and brains dissected at 0 and 6 h after initiation of the stressor.

We analyzed luciferase activity in tadpole brain homogenates using the dual-luciferase reporter assay system (Promega). We processed each brain separately in a Dounce homogenizer in 20 μl of passive lysis buffer, incubated on ice for 30 min with vortexing every 10 min, and then analyzed 10 μl of each homogenate in the dual-luciferase assay.

Statistical analysis

Statistically significant differences were determined using unpaired Student's *t* test or one-way ANOVA followed by Fisher's least significant difference (LSD) multiple comparisons tests. Data were $\log_{10}$ transformed when the variances were found to be heterogeneous. The values are presented as mean + SEM, and $P < 0.05$ was considered statistically significant.

Results

Expression and organization of the X. laevis CRF genes

Using RT-PCR, we found that both CRF genes are expressed in *X. laevis* brain; the identities of the PCR products were confirmed by DNA sequencing. With RT-qPCR we found that xCRFb hnRNA is approximately 10 times more abundant than xCRFa hnRNA when analyzed in whole brain or just in the POA/hypothalamic region (data not shown).

We sequenced the entire 5′ flanking regions represented within genomic clones of two *X. laevis* CRF genes, which include approximately 4.5 kb of CRFa and 1.3 kb of CRFb upstream sequence from the respective ATG translation start sites (GenBank accession no. DQ865136 and DQ865137) (20). Alignment of partial cDNA sequences (obtained by sequencing of PCR products and cDNA library screening; data not shown) with the genomic sequences revealed that the two frog CRF genes, like mammalian CRF genes, possess two exons and a single intron. We aligned the upstream regions of the frog and human CRF genes, which allowed us to assign provisional transcription start sites for the frog genes. Based on these criteria, the xCRFa gene is composed of a first exon of 141 bp, followed by an intron of 697 bp, and the beginning of the protein coding region (AUG codon) is 93 bp downstream of the start of the second exon. The xCRFb gene is composed of a first exon of 162 bp, an intron of 410 bp, and the AUG codon is 55 bp downstream of the start of the second exon. The highest degree of sequence similarity between the two frog CRF genes was found in the coding regions, with 95% sequence identity. The first exon of the two *X. laevis* CRF genes that contain most of the 5′ untranslated regions share 70% sequence identity, whereas their introns are 65% identical.

Structural analysis of the 5′ regulatory regions of X. laevis CRF genes

An earlier study by Vamvakopoulos *et al.* (43) revealed a high degree of sequence similarity (94%) among the first 334 bases upstream of the transcription start sites in the human and ovine CRF genes, but the sequence similarity dropped to 72% approximately 0.6 kb upstream of this region (we compared human and rat and found 94% similarity in the proximal 334 bases, and 67% ~0.6 kb upstream; Yao, M., unpublished data). Because all of the characterized transcription factor binding sites in the mammalian CRF promoters are located within the first 330 bp of 5′ flanking sequence, we focused on these regions of the frog CRF genes for our initial promoter analyses.

Comparison of the 5′ proximal sequences of the two frog CRF genes (325–345 bp upstream of the transcription start sites) revealed approximately 75% sequence similarity. Within the first approximately 340–360 bp of the 5′ flanking region, the human CRF gene shares 63 and 72% sequence similarity with the xCRFa and xCRFb genes, respectively (Fig. 1). A search for transcription factor binding sites in the 5′ flanking regions of the frog genes revealed a putative CRE, a NGFI-B response element, several AP1 sites, and half GREs. All of these elements and their flanking sequences in the frog share very high degrees of similarity with those identified in human and rat CRF genes (Fig. 1) (4, 5, 9).

Exposure to a physical stressor activates transcription of the CRF gene and increases CRF and pCREB immunoreactivity in the frog POA

We exposed juvenile frogs to shaking/handling stressor for 1 h and analyzed expression of CRF genes in the POA/hypothalamic region by RT-qPCR. We designed specific Taqman assays that targeted the intronic regions of the xCRFa and xCRFb genes to analyze expression of the primary tran-

scripts (hnRNA). The 5′ UTR and coding regions of the mRNAs of the two frog CRF genes are highly conserved; thus, the single CRF mRNA Taqman assay detected mature transcripts for both genes. We found that the hnRNA of xCRFb increased significantly ($P = 0.02$) after 1 h exposure to shaking/handling (Fig. 2A), However, we saw no change in xCRFa hnRNA (data not shown). Also, there was no significant change in CRF mRNA after 1 h exposure to the stressor (Fig. 2A).

We then exposed juvenile frogs to shaking/handling stressor for 20 min, 1 h, and 2 h and analyzed CRF-ir and pCREB-ir throughout the brain. In all cases the subcellular location of CRF-ir was predominantly cytosolic, whereas pCREB-ir was exclusively nuclear. In unstressed frogs, pCREB-ir-positive neurons were found throughout the brain (data not shown). Exposure to shaking/handling stressor caused a significant increase in CRF-ir in the POA by 1 h that was maintained through 2 h (1 h, $P = 0.037$; 2 h, $P = 0.013$; n = 3/treatment; Fig. 2, B and C). The stressor produced a rapid (by 20 min) increase in the number of pCREB-ir nuclei in the POA, which was maintained through 2 h (Fig. 3A). Morphometric analysis revealed that exposure to the stressor produced significant increases in the number of pCREB-ir cells in the POA at all time points examined (20 min, $P = 0.047$; 1 h, $P = 0.026$; 2 h, $P = 0.014$. n = 3/treatment; Fig. 3B).

Using dual-immunofluorescence detection coupled with confocal microscopy, we found that in the POA, the majority of the CRF-ir-positive cells are also pCREB-ir positive, whereas not all pCREB-ir-positive cells express CRF-ir (data not shown).

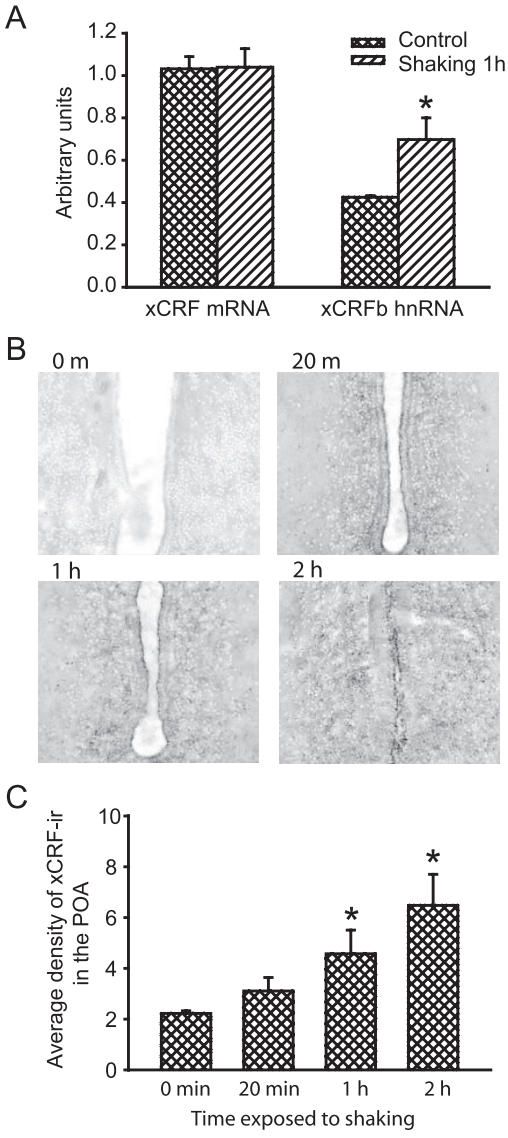

FIG. 2. A, Analysis of CRF mRNA and xCRFb hnRNA in the anterior POA/hypothalamus of juvenile frogs by RT-qPCR. Frogs were either left undisturbed (unstressed) or exposed to 1 h shaking/handling stressor. B, Changes in CRF-ir in the POA of juvenile *X. laevis* at 0 time, 20 min, 1 h, and 2 h after exposure to shaking/handling stressor. C, Densitometric analysis shows the mean density of CRF-ir was significantly increased in the POA at 1 and 2 h in stressed *X. laevis*, compared with animals at 0 time (unstressed controls) and 20 min. Data presented are the mean ± SEM. *, Significant difference from unstressed controls, $P < 0.05$.

The proximal frog CRF promoters support basal and forskolin-induced transcription in transient transfection assays

We constructed promoter-reporter constructs designated pGL3-xCRFa533 and pGL3-xCRFb576 that contain the 5′ proximal sequences of the frog CRFa and CRFb genes, respectively (see *Materials and Methods*). Both pGL3-xCRFa533 and pGL3-xCRFb576 supported basal transcription in trans-

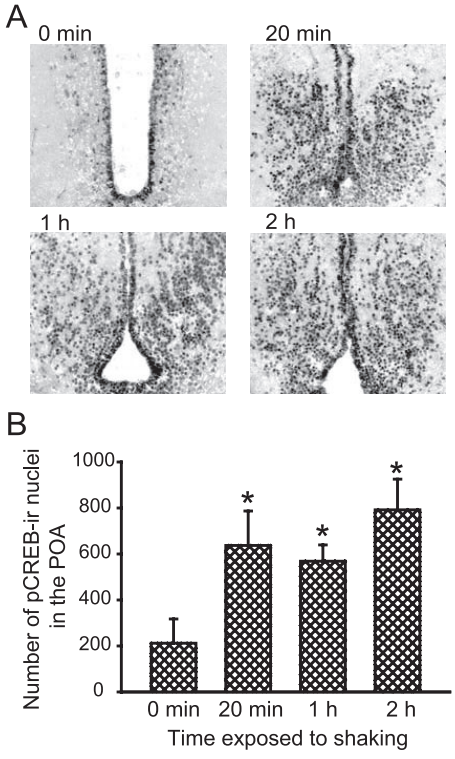

FIG. 3. A, Changes in pCREB-ir in the anterior POA of juvenile *X. laevis* exposed to shaking/handling stressor. B, The total number of pCREB-ir positive nuclei in the POA was significantly increased at 20 min, 1 h, and 2 h in stressed *X. laevis*, compared with unstressed controls. Data presented are the mean ± SEM. *, Significant differences from unstressed controls, $P < 0.05$.

fected PC-12 cells that was 2–3 times that of the parent vector pGL3-basic ($P < 0.05$). Treatment with 25 μM forskolin, an activator of adenylyl cyclase, for 6 h increased luciferase activity by an average of 10-fold for pGL3-xCRFa533 and an average of 43-fold for pGL3-xCRFb576. Luciferase activity in pGL3-basic-transfected cells did not change after treatment with forskolin (Fig. 4A).

We next conducted a time-course experiment to determine an appropriate treatment duration to further analyze forskolin-induced activation of the frog CRF promoters. We focused on the pGL3-xCRFb576 construct that gave a greater response than the pGL3-xCRFa533 at the one time point tested (6 h). Forskolin caused statistically significant promoter activation by 1 h ($P < 0.05$), which continued to increase up to 5 h and was maintained through 7 h (Fig. 4B). By 24 h the luciferase activity remained elevated above untreated, transfected cells and was comparable with the forskolin-induced response at 2 h. Luciferase activity of pGL3-xCRFb576-transfected cells cultured in the absence of forskolin did not change over the 24-h period. Based on these data, we chose 6 h as a routine time point for subsequent analyses. This time point has also been used in transfection experiments with mammalian CRF gene promoter constructs in PC-12 cells (4, 7).

To determine whether forskolin-dependent activation of the frog CRFb gene was mediated by the predicted CRE, we introduced two point mutations into the CRE octomer present in the xCRFb core promoter (changed from TTACGTC*A* to TTA*A*GTC*T*) to create pGL3-xCRFb[CREmut]. Similar point mutations have been shown to eliminate

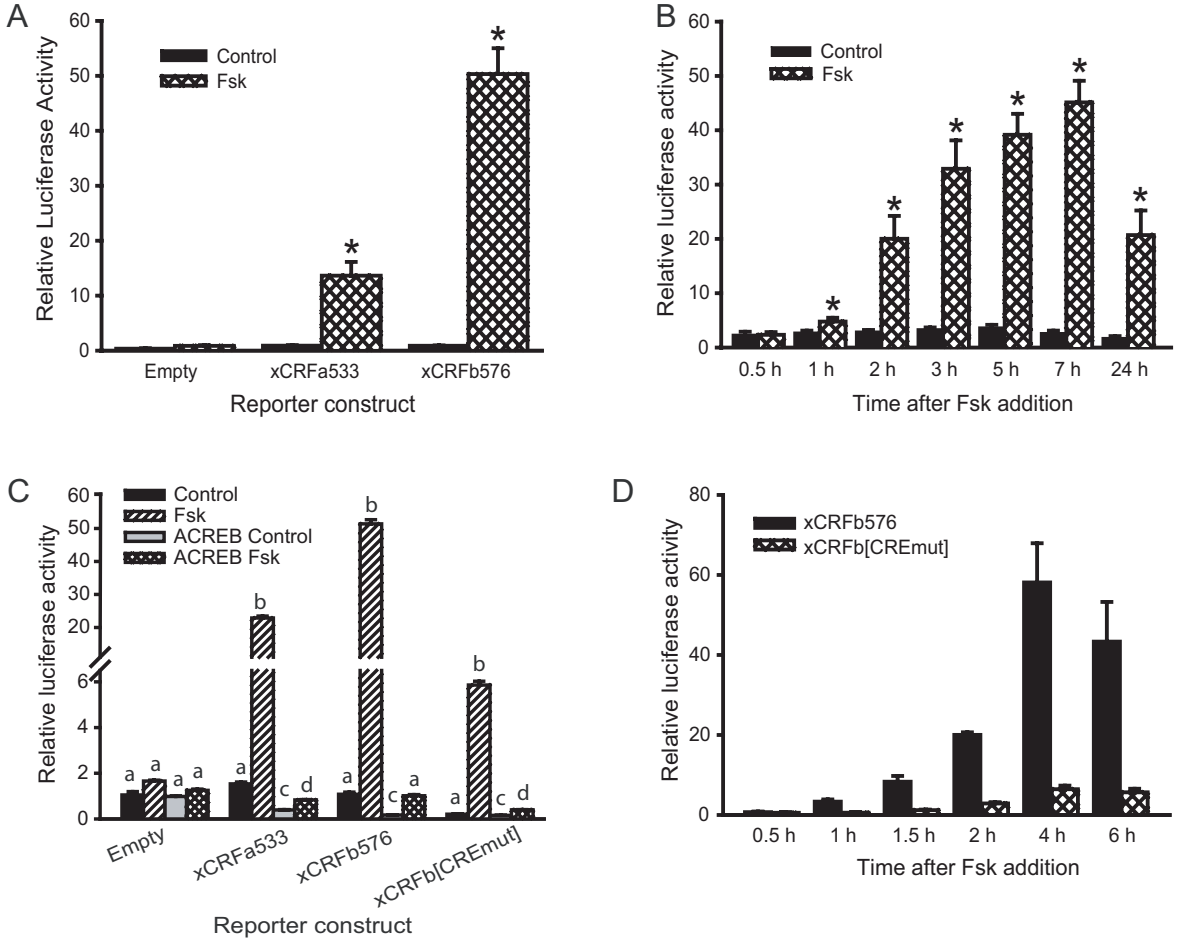

FIG. 4. A, Activation of frog CRF promoter-reporter constructs by 6 h treatment with forskolin (Fsk; 25 μM) in transiently transfected PC-12 cells. PC-12 cells were transfected with empty vector pGL3-basic (Empty), pGL3-xCRFa533 (xCRFa533; 533-bp fragment of the xCRFa promoter), or pGL3-xCRFb576 (xCRFb576; 576-bp fragment of the xCRFb promoter) plus pRL-null to normalize for transfection efficiency (dual luciferase assay; see *Materials and Methods*). The experiment was repeated five times in triplicate, and data (mean ± SEM) of a representative experiment are shown. *, Significant difference, compared with the respective control (Student's *t* tests, $P < 0.05$). B, Time course of activation of pGL3-xCRFb576 by forskolin in transiently transfected PC-12 cells. The construct pGL3-xCRFb[CREmut] (xCRFb[CREmut]) contains the same promoter fragment as the pGL3-xCRFb576 except with two point mutations introduced into the CRE (from TTA*C*GTC*A* to TTA*A*GTC*T*). Transfected cells were treated with or without forskolin (25 μM) for various times. *Bars* represent means ± SEM of two independent experiments conducted in triplicate. *, Significant differences between Fsk-treated and control cells (Student's *t* tests, $P < 0.05$). C, Effect of point mutations introduced into the putative CRE of the xCRFb promoter and cotransfection of a vector expressing ACREB, a mutant form of CREB with dominant-negative activity, on basal activity and the response to forskolin (25 μM; 6 h) in transiently transfected PC-12 cells. The experiment was repeated three times in triplicate, and data (mean ± SEM) of one representative experiment are shown. *Letters* indicate significant differences among treatment means within each reporter construct ($P < 0.05$). D, Effect of point mutations in the putative CRE of the xCRFb promoter on the time course of forskolin (25 μM)-induced activation in transfected PC-12 cells.

forskolin-responsiveness of the human CRF promoter in cell transfection (44). This construct exhibited 63% less basal and 86% less forskolin-induced luciferase activities when compared with the wild-type promoter construct ($P < 0.05$ for both comparisons; Fig. 4C).

These results show that the CRE identified in the xCRFb promoter is functional and mediates gene regulation by the cAMP pathway, presumably through binding of CREB. However, we observed a residual forskolin-induced activation in the pGL3-xCRFb[CREmut] transfected cells. We found similar residual forskolin-dependent responses when we replaced the entire CRE octomer with an unrelated sequence (AGCTAGCT; data not shown), suggesting that it is not due to low affinity binding of CREB at this site (see also EMSA results below). We hypothesized that this activity could be due to a second (or more) CRE or CRE-like element in the CRF promoter or the activation of the cAMP pathway inducing expression of other transcription factors such as c-Fos or NGFI-B. We conducted a time-course experiment with forskolin treatment of cells transfected with the pGL3-xCRFb576 or pGL3-xCRFb[CREmut] constructs. Although the magnitude was much lower, the time course of activation of pGL3-xCRFb[CREmut] paralleled that of pGL3-xCRFb576 with significant elevation ($P = 0.002$) over the no-treatment control by 1.5 h (Fig. 4D; for analysis of the fold increase caused by forskolin treatment of both promoter constructs, also see supplemental data Fig. 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org). The early kinetics are consistent with activation mediated by CREB, although we cannot rule out the involvement of other transcription factors, particularly at later time points.

We next used a dominant-negative approach to further investigate a role for CREB in forskolin-induced CRF promoter activity. We cotransfected PC-12 cells with pGL3-xCRFa533 or pGL3-xCRFb576 and a vector to express a dominant-negative mutant form of CREB (ACREB; Fig. 4C) (45). Cotransfection of ACREB reduced basal promoter activity by 74% (pGL3-xCRFa533) and 84% (pGL3-xCRFb576) and completely blocked forskolin-induced activation of both constructs. Furthermore, ACREB reduced basal activity by 25% and abolished forskolin-induced activity of the pGL3-xCRFb[CREmut]. These data suggest the existence of a second, perhaps lower-affinity CRE-like site in the xCRFb promoter.

As described above, we found that activation of the xCRFb promoter by forskolin was 2- to 3-fold greater than for the xCRFa. We thus sought to test whether this difference in responsiveness was due to the composition of the major CRE sites present in the two core promoters. The xCRFa CRE differs from xCRFb and thus the consensus CRE octomer in that it has a 5′ T→C substitution. We first introduced point mutations to convert the xCRFa CRE into the xCRFb CRE (changed from *c*CTACGTCA to *t*TTACGTCA), thus creating pGL3-CRFa[CREb]. We also mutated the same two nucleotides in the xCRFb CRE to convert it to the xCRFa CRE (changed from *t*TTACGTCA to *c*CTACGTCA), thus creating pGL3-CRFb[CREa]. We found that converting the CRE of the *a* gene to that of the *b* gene (pGL3-xCRFa[CREb]) increased basal promoter activity by 1.7-fold ($P < 0.001$) and forskolin-induced promoter activity by 1.8-fold ($P = 0.002$), compared with that of the pGL3-CRFa533 construct (Fig. 5A; for analysis of the fold increase caused by forskolin treatment, also see supplemental data Fig. 2). Forskolin-induced activation of pGL3-xCRFa[CREb] was of similar magnitude to that of pGL3-xCRFb576. By contrast, converting the CRE of the *b* gene to that of the *a* gene (pGL3-CRFb[CREa]) had no effect on promoter activity, resulting in basal and forskolin-induced promoter activities similar to those of the native promoter construct pGL3-xCRFb576. Thus, the composition of the CRE elements only partly determines the differences in forskolin-induced activity of the two promoters, and other promoter elements also contribute to the responsiveness to forskolin treatment.

PC-12 cells have been used to study mammalian CRF promoter function because they have a functional cAMP pathway, and we found that the frog CRF promoters were functional in these cells (and also in NIH3T3 cells; data not

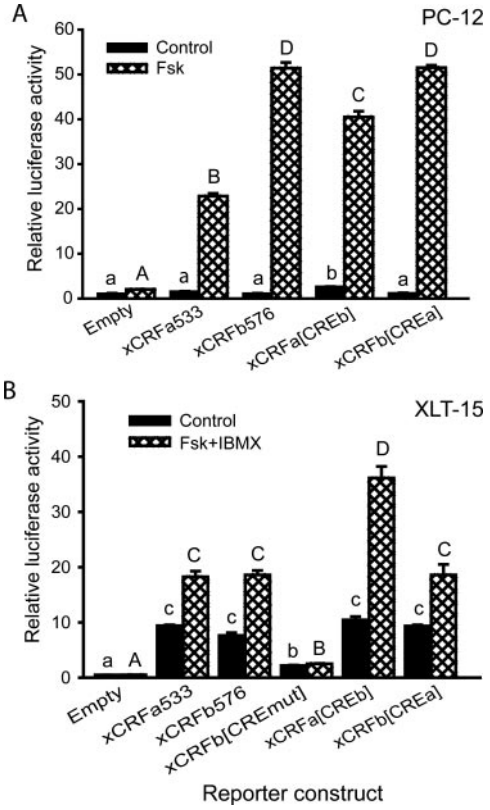

FIG. 5. A, Effects of exchanging the CREs present in the xCRFa and xCRFb genes on basal and forskolin-dependent (Fsk; 25 μM; 6 h) promoter activity in transiently transfected PC-12 cells. The experiment was repeated three times in triplicate, and data from one representative experiment are shown (mean ± SEM). *Letters* indicate significant differences among constructs within a treatment [control, *lowercase*; forskolin, *uppercase*; ANOVA F (9,20) = 1239.5, Fisher's LSD multiple comparisons tests, $P < 0.001$]. B, Basal and forskolin-induced activity (Fsk 25 μM + IBMX 500 nM; 24 h) of frog native and CRE mutant CRF promoter constructs in transiently transfected *X. laevis* tadpole tail myoblast-derived (XLT-15) cells. Shown are the means ± SEM, and *letters* indicate significant differences among constructs within a treatment [control or Fsk + IBMX; ANOVA F (13,28) = 188.7, Fisher's LSD multiple comparisons tests, $P < 0.001$]. Each experiment was repeated three times in triplicate, and data from one representative experiment are shown. All reporter constructs were constructed using the pGL3-basic vector.

shown). To test whether our CRF promoter constructs were functional in frog cells, we used a *X. laevis* tadpole tail myoblast-derived cell line, XLT-15 (36). This cell line expresses CRF and also CRF type 1 receptor (37). We found that both pGL3-xCRFa533 and pGL3-xCRFb576 exhibited significantly higher basal activity in XLT-15 cells, compared with PC-12 cells (Fig. 5B). Treatment of XLT-15 cells with forskolin plus IBMX produced an approximately 2-fold activation of both promoters; there were no differences in the promoter activities among the *a* and the *b* genes in XLT-15 cells. Basal activity of the xCRFb construct was significantly reduced by mutation of the CRE (pGL3-xCRFb[CREmut]; $P < 0.001$, compared with basal activity of pGL3-xCRFb). By contrast to its residual activity in PC-12 cells, we found no increase in promoter activity of the pGL3-xCRFb[CREmut] after forskolin treatment in XLT-15 cells.

Interestingly, we found that changing the CRE in the xCRFa to CREb resulted in a higher induction by forskolin than either of the native promoters (pGL3-xCRFa533 or pGL3-xCRFb576; Fig. 5B). Similar to the results in transfected PC-12 cells, basal and forskolin-induced activities of pGL3-xCRFb[CREa] in XLT-15 cells are similar to those of pGL3-xCRFb576 (Fig. 5B).

CREB binds to the putative CREs present in the frog CRF promoters

Supershifted protein-DNA complexes of similar mobility were formed in EMSA on the CREs from both frog CRF genes using either *in vitro* expressed recombinant *X. laevis* CREB (rxCREB) or frog brain nuclear extract (Fig. 6A). A similar sized complex was formed with the CRE from the human CRF promoter, which contains a palindromic consensus CRE octomer (included as a positive control). We observed little or no binding of rxCREB and no binding of proteins in the nuclear extract to the mutated CRE from the *b* gene (xCRFb[CREmut]; possessing the same point mutations in the CRE as in the pGL3-xCRFb[CREmut] used in transfection) or to the xCRFb[CREdel] probe (Fig. 6A). The specificity of CREB binding to the human and *X. laevis* CRE probes was confirmed by complete displacement of radiolabeled probes by the addition of radioinert oligonucleotides (100 nM) but not the addition of xCRFb[CREmut] or xCRFb[CREdel] (Fig. 6B).

We confirmed the identity of proteins present in frog brain nuclear extract that formed a complex on the CRE probes by antibody supershift. The addition of antiserums to CREB or pCREB to the EMSA reactions supershifted the protein-DNA complexes, thus demonstrating that the major band formed in the EMSA contained CREB or CREB-like proteins (Fig. 6C).

CREB associates with proximal frog CRF promoter in vivo

We used ChIP assay combined with quantitative PCR to determine whether CREB associates with the frog CRF promoters *in vivo*. We prepared chromatin from brain sections of juvenile frogs that included the POA and hypothalamus and conducted ChIP assays for CREB and acetylated histone H4. Nonimmune normal rabbit serum was used as a negative control. Our results show that the ChIP signal for CREB at the CRE region of the xCRFb proximal promoter was significantly higher than at the CDS of the xCRF genes ($P = 0.02$; analysis of the CDS was included as a negative control; Fig. 7A). We also observed significantly higher acetylated histone H4 at the CRE region, compared with the CDS of the xCRF genes ($P < 0.001$; Fig. 7B).

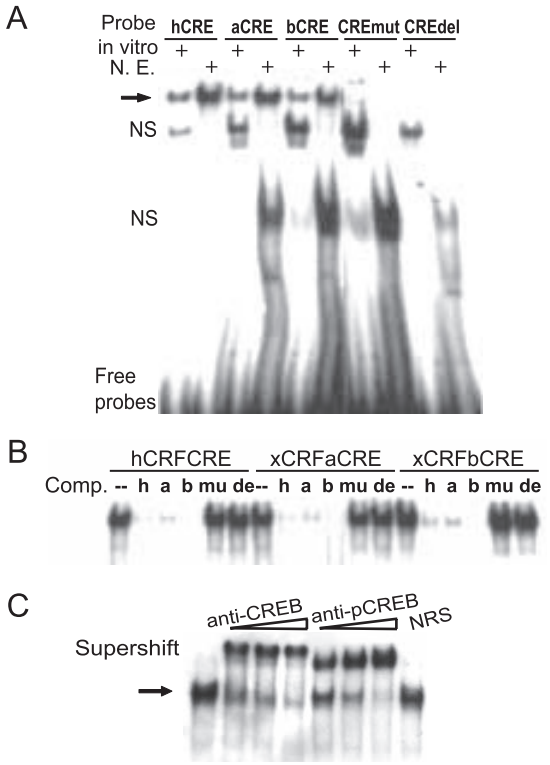

FIG. 6. A, Binding of rxCREB or nuclear extracts derived from the anterior POA/hypothalamus of the juvenile frog brain (see *Materials and Methods*) to ^{32}P-labeled CRE probes as analyzed by EMSA. Sequences of the probes are given in Table 1. Lanes 1, 3, 5, 7, and 9 incubated with rxCREB; lanes 2, 4, 6, 8, and 10, incubated with brain nuclear extract (N.E.). The major band is indicated by an *arrow*. NS, Nonspecific bands. B, Cross-competition of CREB binding to ^{32}P-labeled CRE probes by radioinert oligonucleotides [competitor (Comp.)]. The ^{32}P-labeled hCRFCRE, xCRFaCRE, or xCRFbCRE probes were incubated with brain nuclear extract in the presence or absence of 100 nM of radioinert competitor oligonucleotides: −, no competitor; h, hCRFCRE; a, xCRFaCRE; b, xCRFbCRE; mu, xCRFb[CREmut]; de, xCRFb[CREdel]. C, Antibody supershift EMSA using antiserum to CREB (anti-CREB), phosphorylated CREB (anti-pCREB), or NRS. The ^{32}P-labeled xCRFbCRE probe was incubated with frog brain nuclear extract in the presence or absence of varying amounts of serum (1, 2, and 4 μl of anti-CREB; 0.25, 0.5, and 1 μl of anti-pCREB; 10 μg of NRS IgG). The specific CREB-DNA complex is indicated by the *arrow*.

After exposure to shaking/handling stressor for 1 h, we observed significantly greater association of CREB at the CRE region of the xCRFb promoter ($P = 0.026$). There were no changes in CREB association in the CDS of the CRF genes after exposure to the stressor (Fig. 7C) nor at the proximal promoter of the xCRFa gene (data not shown).

Analysis of CRF promoter activity in transfected X. laevis brain in vivo

To test the functionality of the frog CRF promoter-luciferase reporter constructs *in vivo*, we transfected brains of NF stage 50 tadpoles by electroporation-mediated (EM) gene

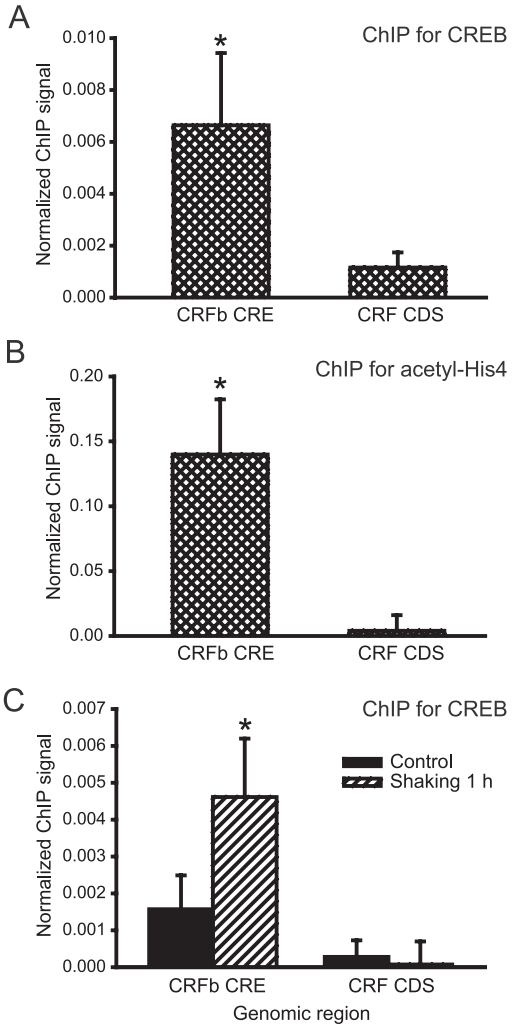

FIG. 7. Association of CREB or acetylated histone H4 with the CRE region of the CRFb proximal promoter as analyzed by ChIP assay. ChIP assays for CREB (A) or acetylated histone H4 on chromatin (B) were obtained from brain of unstressed frogs. C, ChIP assay for CREB on chromatin obtained from brain of frogs exposed to shaking stressor for 1 h. The genomic regions analyzed were the proximal promoter of xCRFb targeting the CRE (CRFb CRE), the coding regions of xCRF genes (CRF CDS; assay did not distinguish the two genes; considered a negative control). Ten hypothalamic sections derived from juvenile frogs were pooled for each sample, and three to four replicate pools were analyzed for each treatment. Data are expressed as normalized ChIP signal. That is, the values for each sample obtained by ChIP with anti-CREB or anti-acetyl-histone H4 were first divided by the respective input values, followed by subtraction of the values obtained by ChIP using NRS (background signal). Shown are means ± SEM. Derived values were $\log_{10}$ transformed before ANOVA followed by Fisher's LSD multiple comparisons tests. *, Significant difference from the coding regions of xCRF (CRF CDS; A and B) or unstressed control (C) ($P < 0.05$).

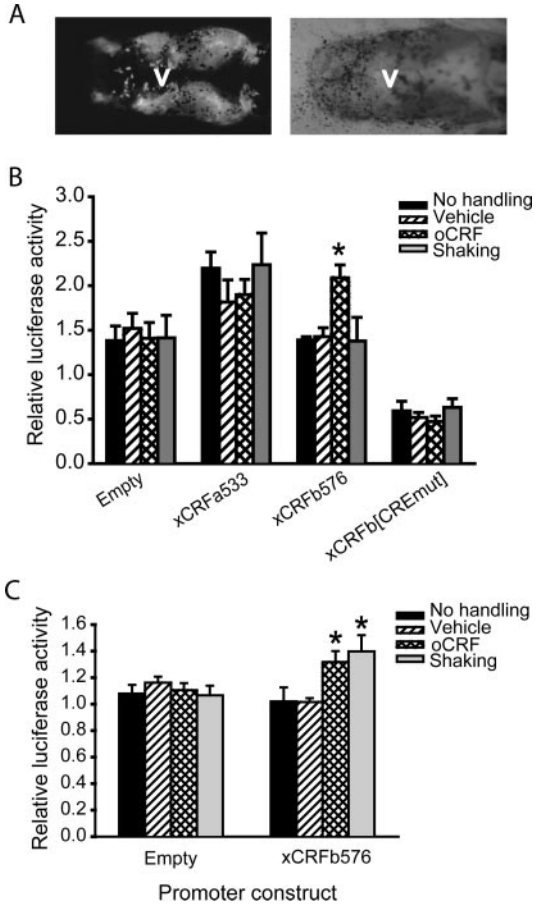

FIG. 8. A, Fluorescent (*left panel*) and light (*right panel*) microscopic images of the brain of a tadpole transfected with pEGFP-N1 plasmid by electroporation. V, Third ventricle. B, Effects of i.c.v. injection of oCRF or exposure to shaking/handling stressor on the activity of CRF promoter constructs transfected into tadpole brain by EM gene transfer. Tadpoles were electroporated with the indicated constructs (all constructed using the pGL3-basic vector) plus pRL-null to control for transfection efficiency (see *Materials and Methods*) and 1–2 wk later given i.c.v. injections of either saline or oCRF (20 ng/g body weight) or exposed to shaking/handling stressor. Animals were killed 6 h after injection or initiation of the shaking/handling stressor and brains collected for dual luciferase assay. C, Effects of i.c.v. injection of oCRF (20 ng/g body weight) or exposure to shaking/handling stressor on the activity of CRF promoter constructs in brains of juvenile frogs (body weight 0.6–2.5 g). Plasmids were transfected into premetamorphic tadpole brain by EM gene transfer, and the animals were raised to 2–3 wk after metamorphosis. All animals were killed 6 h after i.c.v. injection or initiation of the shaking stressor. Each experiment was repeated twice, and data from a representative experiment are shown as the mean ± SEM. Sample sizes are eight to 19 (B) and six to eight (C) for each treatment group. *, Significant differences, compared with the respective no injection/no handling controls within a construct ($P < 0.05$).

transfer. We used two approaches to control for transfection efficiency. First, we cotransfected the pEGFP-N1 vector and screened tadpoles for EGFP expression 5 d later. We examined the animals under a fluorescent stereomicroscope and selected those with high and comparable EGFP fluorescence in the region of the third ventricle (~75% of electroporated tadpoles; an example is shown in Fig. 8A). Second, we cotransfected a promoterless *Renilla* luciferase vector, which allowed us to conduct a dual-luciferase assay on tissue homogenates from transfected animals. We thus normalized the firefly luciferase activity (which was under the regulation of the CRF promoters) to the *Renilla* luciferase.

We analyzed basal and induced expression of the xCRFa533, xCRFb576, and xCRFb[CREmut] constructs in tadpole brain (Fig. 8B) but only the xCRFb576 construct in juvenile frog brain (Fig. 8C; animals were transfected as tadpoles and then grown to the juvenile frog stage). In the

tadpole, the basal activity of pGL3-xCRFb576 was indistinguishable from the parent vector pGL3basic, whereas activity of pGL3-xCRFa533 was significantly higher than pGL3-basic and pGL3-xCRFb576 (Fig. 8B; $P < 0.001$). By contrast, activity of the pGL3-xCRFb[CREmut] was significantly lower, compared with pGL3-basic ($P = 0.011$). To test for inducible activity of the frog CRF promoter constructs *in vivo*, we injected oCRF i.c.v. (20 ng/g body weight) and killed animals 6 h later for dual-luciferase assay. oCRF binds to and activates the frog type 1 CRF receptor resulting in elevations of intracellular cAMP and thus activation of the cAMP pathway (46). We chose to use oCRF because it does not bind to the frog CRF binding protein, and thus, its activity would not be abrogated by such an interaction (47). We found that oCRF caused a significant increase in luciferase activity in brain homogenates from pGL3-xCRFb576-transfected animals, compared with no-injection or saline-injected controls ($P = 0.002$). The same treatment did not alter the activity of pGL3-basic, pGL3-xCRFa533, or pGL3-xCRFb[CREmut]. Saline injection did not alter the expression of any of the constructs, compared with the respective no-injection controls. We also subjected the transfected tadpoles to shaking/handling stressor for 6 h but observed no significant changes in luciferase activity with any of the constructs, compared with the no-handling controls (Fig. 8B).

We raised a subset of transfected tadpoles through metamorphosis and analyzed promoter activity in juvenile frogs. Luciferase activity in brain homogenates of transfected animals was detectable at the juvenile stage, although it was significantly lower, compared with activity in the tadpole stage. We first injected oCRF i.c.v. and measured luciferase activity 6 h later. Similar to the results in transfected tadpoles, oCRF caused a significant increase in luciferase activity in pGL3-xCRFb576 transfected frogs, compared with no-injection ($P = 0.033$) or saline-injected controls ($P = 0.005$); the same treatment did not alter activity in pGL3-basic-transfected animals. Injection of saline did not alter the expression of any of the constructs, compared with the no-injection controls (Fig. 8C).

We also subjected frogs transfected with pGL3-xCRFb576 to shaking/handling stressor for 6 h, and by contrast to the transfected tadpoles, we observed significantly increased luciferase activity, compared with no handling controls ($P = 0.03$; Fig. 8C; i.c.v. oCRF was included as a positive control in the experiment). We observed no change in pGL3-basic activity after exposure to the stressor.

Discussion

We discovered that the gene organization and proximal promoter elements of frog CRF genes are remarkably conserved with mammalian CRF genes, two tetrapod lineages that diverged more than 200 million years ago. This high degree of conservation argues that the structural elements responsible for the spatial and physiological regulation of CRF genes arose early in vertebrate evolution and have been maintained by strong positive selection owing to the pivotal role that CRF plays in physiological and behavioral adaptation. Furthermore, our findings provide strong support for the hypothesis that stressor-induced activation of vertebrate CRF genes *in vivo* is dependent on CREB binding to the CRE located in the proximal promoter region.

Earlier we found that the distribution of CRF neurons in the brain of *X. laevis* was highly conserved with that of mammals, and that CRF-ir was strongly increased in the anterior preoptic area (homologous to the mammalian paraventricular nucleus), medial amygdala, and bed nucleus of the stria terminalis after exposure to shaking/handling stressor (21). The similarity of the central distribution of frog CRF neurons to mammals, and their stressor-dependent activation suggested the existence of conserved gene-regulatory mechanisms. Mammalian CRF genes exhibit a very high degree of conservation (94%) within the proximal promoter regions (first 330-bp flanking sequence) (Yao, M., unpublished data) (43). We found that the frog CRF promoters (~340-bp flanking sequence) are also very highly conserved with mammalian genes, exhibiting 63% (xCRFa) and 72% (xCRFb) similarity with the corresponding region of the human CRF gene.

The overall sequence similarity between the frog CRFa and CRFb genes within the approximately 340 bp upstream of the transcription start site is 75%, which is comparable with the sequence similarity between either of the frog genes and human gene. Thus, the duplicate frog CRF genes have been diverging at a faster rate than CRF genes among mammalian species. The proximal promoter region of xCRFb is more highly conserved with mammalian genes than xCRFa, suggesting that the *a* gene may be diverging at a faster rate than the *b* gene. This divergence may explain the low responsiveness of the xCRFa promoter to forskolin treatment in transfected cells and its lack of activation by i.c.v. CRF injection in transfected tadpole brains. Furthermore, data from both semiquantitative and quantitative RT-PCR analysis of hnRNA suggest that the xCRFb gene is expressed at a higher level than the xCRFa gene in the frog brain (Yao, M., and R. J. Denver, unpublished data). This may mean that the *a* gene is becoming a pseudogene. Alternatively, the *a* gene may be acquiring a novel functional role based on the modification of its regulatory regions. The conservation of the coding regions of the two frog CRF genes suggests that there is selection to maintain the structure of the mature CRF peptides but divergent evolution in the regulatory regions of the two genes.

Computer analysis of the frog genes revealed several putative transcription factor binding sites in the proximal promoter region that are conserved with mammalian genes. These included consensus CRE, NGFI-B response element, AP1, and GRE sites that have been implicated in CRF gene regulation in mammals. We also have evidence that the AP1 and GRE sites in the frog CRF promoters are functional (Yao, M., and R. J. Denver, unpublished data). However, in the present study, we focused on the role of the putative CRE sites, and we provide several lines of evidence that support the functionality and essential nature of the CREs in the frog genes for gene activation by the cAMP pathway *in vivo*.

Several studies in mammals have shown that CREB is rapidly phosphorylated and thus activated in CRF neurons in response to various stressors, preceding an elevation of CRF expression in the same cells (16, 18, 48). We found that in juvenile frogs, pCREB-ir was significantly increased in the

POA after 20 min exposure to shaking/handling stressor, which is consistent with the early kinetics of pCREB accumulation observed in mammals. To our knowledge, this is the first demonstration of stressor-dependent CREB activation in the brain of a nonmammalian vertebrate. In the frog as in the mammal, we observed a delay in the increase of CRF-ir in the same cells that were immunopositive for pCREB, occurring 1 h after exposure to the stressor. The early appearance of pCREB in CRF neurons of the POA show that pCREB is present in these neurons and thus could mediate stressor-dependent activation of frog CRF genes *in vivo*.

We used transient transfection assays in PC-12 cells to determine whether the frog CRF promoters support basal transcription and whether the putative CREs present in the genes are functional. Both frog CRF promoters exhibited basal activities that were 2- to 3-fold that of the empty vector control. The activity increased after treatment with forskolin by 10-fold for xCRFa and 43-fold for xCRFb. We also determined basal and forskolin-induced CRF promoter activity in a frog cell line, the myoblast-derived XLT-15, which expresses CRF (37). Both promoter constructs showed much higher basal activity in XLT-15 than PC-12 cells (15–19 times the basal activity of the empty vector). We found similar activation of both promoters by forskolin in the frog cells, although to a lesser degree than in PC-12 cells (about 2-fold). These results show that the frog CRF promoters support basal transcriptional activity and activation by the cAMP pathway.

Specific roles for the putative CREs in the frog CRF genes are supported by mutagenesis experiments, in which we found that mutation of the CRE in the xCRFb promoter significantly reduced both basal and forskolin-induced promoter activity in PC-12 cells and completely eliminated forskolin induction in XLT-15 cells. Expression of a dominant-negative CREB strongly reduced basal and eliminated forskolin-induced activity. In further support of the frog CREs being *bona fide* CREB binding sites, we showed by EMSA that recombinant *Xenopus* CREB bound both CREs *in vitro*. The specificity of this binding was demonstrated by displacement with radioinert CRE probes and the failure of the protein to bind to the mutant CREB. Using antibodies to both CREB and pCREB, we showed that the protein-DNA complex formed with frog brain nuclear extract and the CREb probe in EMSA contained predominantly CREB. These data strongly support the hypothesis that CREB binds to the identified CREs in the frog genes and plays an important role in regulating both basal and cAMP-dependent CRF promoter activity. The mutagenesis and dominant-negative CREB experiments suggest that the identified CREs mediate the majority, if not all, of the cAMP-dependent activity.

The frog CRFa promoter exhibited consistently lower forskolin-induced activity than the CRFb promoter in PC-12 cells, both in terms of the absolute level of luciferase and the fold increase above basal caused by forskolin (see Fig. 4A). We hypothesized that this difference was due to sequence variation among the CREs of the two genes and thus tested this by exchanging their CREs through site-directed mutagenesis. Mutating the CRE of the *a* gene into the CREb elevated the absolute level of both basal and forskolin-induced activity in PC-12 cells, compared with the native xCRFa promoter (see Fig. 5A). However, the fold increase was not significantly changed from the native CRFa promoter (see supplemental data Fig. 2). By contrast, swapping the CREb into the *a* gene did not alter the absolute magnitude of basal or forskolin-induced activity or the fold increase. These data suggest that the structure of the CRE can influence the absolute level of both basal and cAMP-dependent promoter activity. However, it is clear that other, as-yet-uncharacterized elements in the core promoters of the two genes also contribute to the differences in activity.

By contrast to PC-12 cells, in the frog cell line XLT-15 we found that replacing the CRE of the *a* gene with the CREb resulted in higher forskolin-induced activity than both native promoters. On the other hand, changing the CRE of the *b* gene to that of the *a* gene did not affect xCRFb promoter activity in XLT-15 cells. Taken together, our data suggest that the core sequence of the CRE consensus site (octomer) is crucial to the basal activity and the responsiveness of the promoters to cAMP pathway activation, whereas other promoter elements also play a significant role in the functionality of the element. Furthermore, promoter activity is dependent on the cell type in which the gene is expressed, which likely reflects cell-specific expression of phosphodiesterase, second messenger molecules, transcription factors, *etc.*

To determine whether CREB occupies the proximal frog CRF promoters *in vivo*, we conducted ChIP assay on chromatin prepared from diencephalon (POA/hypothalamus) of juvenile frogs. This assay confirmed that CREB associated with the CRE region of a frog CRF gene *in vivo* (only the xCRFb gene was analyzed), as has been shown for the CRF gene in the central nervous system of the rat (19). This result is in agreement with a genome-wide analysis conducted on human and rat cell lines, which suggested that CREB occupies a majority of CRE sites located near transcription start sites (49, 50). After cAMP activation CREB is phosphorylated at a conserved serine (Ser-133), which leads to recruitment of the coactivator CREB-binding protein/p300, which has histone acetyltransferase activity and thus promotes transcriptional activation of target genes (51–54). Consistent with this model, we found that the xCRFb proximal promoter had significantly higher acetylated histone H4 than the CDS region. We also found increased CREB association with the xCRFb proximal promoter after exposure to shaking/handling stressor, suggesting that the recruitment of CREB is enhanced by exposure to a stressor. Increased association of CREB with the CRF promoter after activation of the cAMP pathway has been observed in cultured cells (55–57). Shepard *et al.* (19) used ChIP assay to show that the pCREB signal at the CRF promoter in rat brain increased after exposure to a stressor.

The findings discussed above support the view that the CREs present in the proximal promoters of vertebrate CRF genes mediate stressor-dependent activation through CREB binding. In the current study, we used EM gene transfer into frog brain to test whether the CRE is required for CRF gene activation in an *in vivo* context. *In vivo* EM gene transfer has been used for different purposes including the mapping and study of *cis*-regulatory elements (42, 58–62). We used bulk EM gene transfer to transfect a majority of cells surrounding

the third ventricle of the tadpole brain. This method transfects diverse cell types in the brain, and thus, we do not know whether the measured luciferase activity reflects CRF neuronal activity *per se*. However, this approach provides a means for testing whether the CRF promoter constructs, which are functional in cultured cells, can support transcription in cells of a live animal.

Using EM gene transfer, we found that activity of the *b* promoter (pGL3-xCRFb576) was increased significantly in brains of transfected tadpoles by i.c.v. injection of CRF. This showed that the promoter can be activated *in vivo* by an agent that induces cAMP pathway activation. Mutation of the CRE significantly reduced basal activity and abolished promoter activation caused by CRF. We subjected transfected tadpoles to shaking/handling stressor but detected no changes in the activities of any of the constructs. However, when raised to the juvenile frog stage, we found that the activity of pGL3-xCRFb576 was significantly increased by both injection of CRF and shaking/handling stressor. This suggests that the ability of a stressor to activate the CRF promoter may be dependent on the developmental stage, which requires further study. Taken together, our findings with EM gene transfer *in vivo* support *in vitro* cell culture experiments that showed that the CRE in the CRF proximal promoter plays an essential role in both basal and induced gene activity.

Interestingly, although the activity of pGL3-xCRFa533 was significantly higher than pGL3-xCRFb576 *in vivo*, the activity of this construct was not altered by i.c.v. injection of CRF. This finding supports that there are functionally important differences between the regulatory elements of the duplicated genes and that the xCRFa gene may not be inducible *in vivo*.

In conclusion, we provide evidence for an essential role for the CRE present in vertebrate CRF genes for stressor-dependent gene activation. The gene organization and proximal *cis* regulatory elements of CRF genes are highly conserved among frogs and mammals, suggesting that these features of CRF genes were present in the earliest land dwelling vertebrates. Similar responsiveness of fish CRF genes to stressors (63–66) support the view that these regulatory elements arose before the evolution of the tetrapods and have been maintained by strong positive selection owing to the essential nature of the physiological systems controlled by CRF.

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