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Transcriptional Regulation of Pituitary *POMC* Is Conserved at the Vertebrate Extremes Despite Great Promoter Sequence Divergence

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The stress response involves complex physiological mechanisms that maximize behavioral efficacy during attack or defense and is highly conserved in all vertebrates. Key mediators of the stress response are pituitary hormones encoded by the proopiomelanocortin gene (*POMC*). Despite conservation of physiological function and expression pattern of *POMC* in all vertebrates, phylogenetic footprinting analyses at the *POMC* locus across vertebrates failed to detect conserved noncoding sequences with potential regulatory function. To investigate whether ortholog *POMC* promoters from extremely distant vertebrates are functionally conserved, we used 5′-flanking sequences of the teleost fish *Tetraodon nigroviridis POMCα* gene to produce transgenic mice. *Tetraodon POMCα* promoter targeted reporter gene expression exclusively to mouse pituitary cells that normally express *Pomc*. Importantly, transgenic expression in mouse corticotrophs was increased after adrenalectomy. To understand how conservation of precise gene expression mechanisms coexists with great sequence divergence, we investigated whether very short elements are still conserved in all vertebrate *POMC* promoters. Multiple local sequence alignments that consider phylogenetic relationships of ortholog regions identified a unique 10-bp motif GTGCTAA(T/G)CC that is usually present in two copies in *POMC* 5′-flanking sequences of all vertebrates. *Underlined* nucleotides represent totally conserved sequences. Deletion of these paired motifs from *Tetraodon POMCα* promoter markedly reduced its transcriptional activity in a mouse corticotropic cell line and in pituitary POMC cells of transgenic mice. In mammals, the conserved motifs correspond to reported binding sites for pituitary-specific nuclear proteins that participate in *POMC* transcriptional regulation. Together, these results demonstrate that mechanisms that participate in pituitary-specific and hormonally regulated expression of *POMC* have been preserved since mammals and teleosts diverged from a common ancestor 450 million years ago despite great promoter sequence divergence. (*Molecular Endocrinology* 21: 2738–2749, 2007)

THE PROOPIOMELANOCORTIN GENE (*POMC*) encodes a prohormone that gives rise to several bioactive peptides including ACTH, the melanocortins α-, β-, and γ-MSH, and the opioid peptide β-endorphin. *POMC* is mainly transcribed in discrete populations of brain neurons and in the pituitary gland, where it is expressed in melanotrophs of the intermediate lobe and corticotrophs of the anterior lobe. ACTH is a principal component of the hypothalamic-pituitary-adrenal (HPA) axis that mediates the stress response in all vertebrates (1–3). Stress-induced release of the hypothalamic peptide CRH stimulates the release of ACTH from pituitary corticotrophs, which in turn promotes glucocorticoid release from the adrenal gland cortex. As part of a negative feedback loop, glucocorticoids decrease the expression of CRH in the hypothalamus and *POMC* in pituitary corticotrophs. The crucial importance of ACTH in the stress response can be appreciated in humans carrying mutations that inactivate the *POMC* gene, who die from adrenal insufficiency unless permanently supplemented with glu-

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Abbreviations: CHO, Chinese hamster ovarian; HPA, hypothalamic-pituitary-adrenal; Nurr1/NR4A2, nuclear receptor subfamily 4, group A, member 2; Nurr77/NR4A1, nuclear receptor subfamily 4, group A, member 1; PFA, paraformaldehyde; Pitx1, paired-like homeodomain 1; POMC, proopiomelanocortin; PP1, putative pituitary POMC-1; PPCE, pituitary POMC conserved element; Tpit, pituitary-restricted transcription factor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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cocorticoids (4, 5). Although *POMC* is expressed in the pituitary and brain of all species studied to date, several mutations that modify the peptide repertoire of the POMC prohormone have been selected and fixed in different vertebrate lineages. For example, POMC in rodents lacks a di-basic site necessary for a proteolytic cleavage that releases β-MSH (6). In addition, *POMC* sequences that code for γ-MSH were deleted from teleost fish genomes more than 320 million years ago (7, 8).

Transcriptional regulation of pituitary *POMC* has been investigated in several expression systems. Transgenic mouse studies revealed that 400 bp of the rat *POMC* proximal promoter direct pituitary cell-specific and hormonally regulated expression to corticotrophs and melanotrophs (9). In addition, extensive expression studies performed in the mouse corticotropic-derived cell line AtT20 led to the characterization of several *cis*-acting sequences that participate in the transcriptional control of pituitary *POMC*, including binding sites for the following transcription factors: paired-like homeodomain 1 (Pitx1), pituitary-restricted transcription factor (Tpit), Ikaros, neurogenic differentiation 1 (NeuroD1)/β2, Nurr1/NR4A2 (nuclear receptor subfamily 4, group A, member 2), Nur77/NR4A1 (nuclear receptor subfamily 4, group A, member 1), signal transducer and activator of transcription 3 (STAT3), and glucocorticoid receptor (10–15). More recently, 415 bp of the zebrafish proximal *POMC* promoter have also been shown to direct specific expression of a reporter gene to the pituitary of transgenic zebrafish (16).

Despite the similar expression patterns of *POMC* in all vertebrates (7), phylogenetic footprinting analyses at the *POMC* locus of most vertebrate classes failed to detect conserved sequences with potential regulatory function (17). This might be explained by pituitary *POMC* expression being controlled by different transcription factors in different vertebrate classes or, alternatively, because transcription factor binding sites in the ortholog *POMC* promoters are only marginally conserved at the nucleotide sequence. To investigate whether ortholog *POMC* promoters from extremely distant vertebrates are functionally conserved despite their high level of sequence divergence, we tested the ability of 5′-flanking sequences of the *POMCα* gene of the teleost fish *Tetraodon nigroviridis* to direct the expression of a reporter gene to POMC cells of transgenic mice. The *Tetraodon* genome has been sequenced and assembled (18), and it constitutes an advantageous fish model for promoter expression studies because it has one of the most compact vertebrate genomes. Here, we report that pituitary-specific and hormonally regulated expression of *POMC* has been preserved since mammals and teleost fishes diverged from a common ancestor almost half a billion years ago (19). By using a bioinformatic algorithm that takes into account phylogenetic relationships, we detected a conserved 10-bp relic that is present in *POMC* promoters of all vertebrate classes analyzed and proved to be essential for *POMC* promoter activity.

RESULTS

Cell-Specific Expression of Pituitary *POMC* Is Conserved at the Vertebrate Extremes

To investigate whether the *POMC* promoter of a teleost fish is functionally conserved in a mammalian expression system, we sought to study the expression of *Tetraodon POMC* gene constructs in transgenic mice. *Tetraodon*, like other teleost fishes, has two ancient *POMC* paralogs, *POMCα* and -β (8). We chose to study the expression of *POMCα* because it is the most similar to mammalian *POMC* at the sequence level and is strongly expressed in the pituitary gland (8). By screening a fosmid genomic *Tetraodon* DNA library, we isolated a 36-kb fragment that contains the complete *POMCα* transcriptional unit together with the 5′ and 3′ intergenic regions and portions of the upstream and downstream flanking genes. We used this genomic fragment to investigate the expression pattern of the entire unmodified *Tetraodon POMCα* locus in transgenic mice (transgene 1, Fig. 1). *In situ* hybridization experiments using a highly specific and sensitive riboprobe against *Tetraodon POMCα* exon 2 (8) failed to detect transgene 1 expression in the pituitaries or brains of F1 transgenic mice of eight independently generated pedigrees. RT-PCR experiments performed on transgenic pituitaries, hypothalami, and various other tissues also failed to detect *Tetraodon POMCα* mRNA even using various combinations of primers (data not shown). Also, transgene 1 was transcriptionally silent in transient transfection experiments in the mouse corticotropic cell line AtT20, as tested by extensive RT-PCR analyses (data not shown). Because it has been reported that unmodified fish genomic transgenes may not be efficiently expressed or processed in mouse cells (20, 21), we decided to construct fusion transgenes. We first ligated a fragment containing the entire 5′-flanking region of *Tetraodon POMCα*, exon 1, and most of intron 1 to the minimal chicken β-globin promoter fused to the reporter gene *lacZ* and generated transgenic mice (transgene 2; Fig. 1). Analysis of F1 transgenic mice carrying transgene 2 showed β-galactosidase expression in the intermediate and anterior lobes of the pituitary gland in two of five independently generated transgenic pedigrees (Fig. 2). Double histochemistry in pituitary sections demonstrated colocalization of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining in pituitary cells labeled with an anti-ACTH antibody (Fig. 2). Although not all ACTH-expressing cells stained with X-Gal, β-galactosidase activity was observed only within POMC cells. These results demonstrate that the 5′-flanking region of *Tetraodon POMCα* contains *cis*-regulatory elements recognized by the transcription factor machinery that nor-

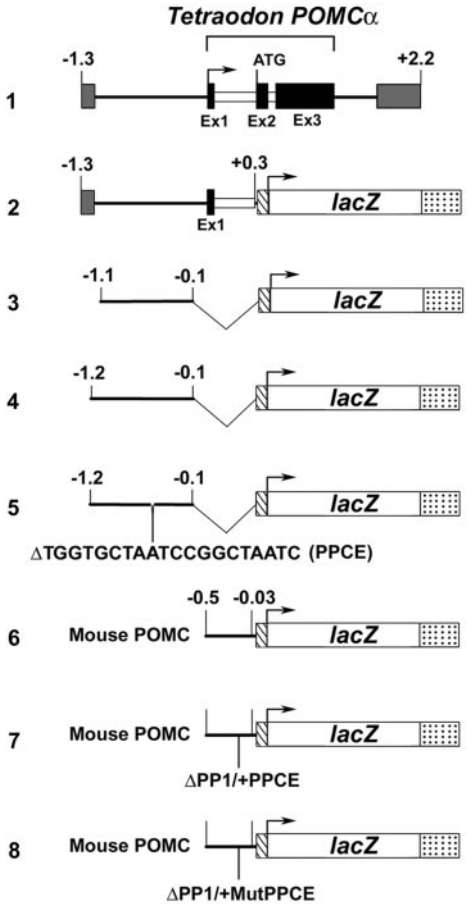


Fig. 1. Transgenes Used in this Study

Transgene 1 contains the complete *Tetraodon POMCα* gene together with 1.3 kb of 5′-flanking region that includes part of the upstream gene (NOD/Caterpiller family; *gray box* at the *left*) and 0.9 kb of 3′-flanking region including part of the downstream gene (KIAA0953 gene homolog; *gray box* at the *right*). The three *Tetraodon POMCα* exons are shown in *black* and introns in *white*. The starting ATG is located in exon 2. Transgenes 2, 3, 4, and 5 include different lengths of the *POMCα* 5′-flanking region cloned upstream of the chicken β-globin minimal promoter (*striped boxes*) followed by the reporter gene *lacZ* (not drawn to scale) and the SV40 polyadenylation signal (poly A, *dotted boxes*). Transgene 5 is identical to transgene 4 except for lacking the conserved element PPCE. Transgene 6 contains the mouse *POMC* proximal enhancer region (from −503 to −31 bp; it does not include the TATAA box). Transgenes 7 and 8 are identical to transgene 6 except that the PP1 site was replaced with PPCE or a mutated PPCE, respectively.

mally transactivates *Pomc* in mouse pituitary cells. Transgenic mice generated with a shorter fragment containing about 1 kb of *Tetraodon POMCα* 5′-flanking DNA gave similar results but with no detectable corticotropic *lacZ* expression under basal conditions (transgene 3; Figs. 1 and 3). None of the transgenic mice expressed transgenes 2 or 3 in the arcuate nucleus of the hypothalamus.

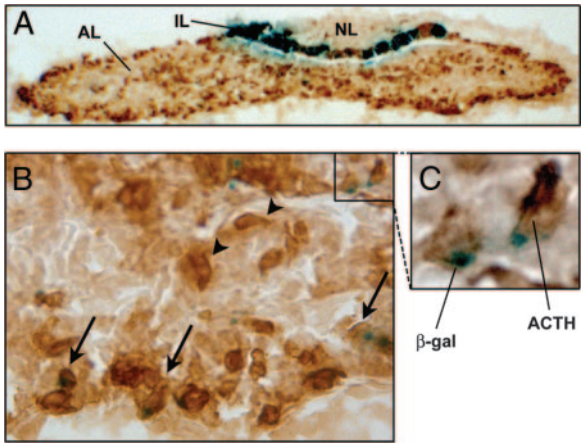


Fig. 2. *Tetraodon POMCα* Promoter Drives β-Galactosidase Expression to Pituitary Corticotrophs and Melanotrophs of Transgenic Mice

A, Coronal section of the pituitary of a newborn (postnatal d 1) mouse transgenic for transgene 2 showing expression of β-galactosidase in corticotrophs of the anterior lobe (AL) and melanotrophs of the intermediate lobe (IL). NL, Neural lobe. B, Higher-magnification image showing colocalization of β-galactosidase (X-Gal staining, *blue dots*) and ACTH immunostaining (diaminobenzidine, *brown*) in corticotrophs (*arrows*). *Arrowheads* show corticotrophs that do not express β-galactosidase. C, *Inset* shows colocalization of β-galactosidase (β-gal, inclusion bodies) and ACTH in corticotroph perikarya.

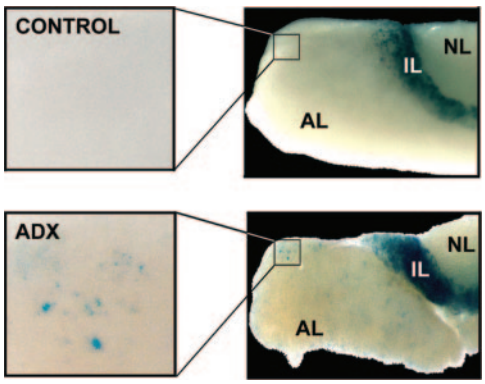


Fig. 3. Mouse HPA Hormonal Changes Regulate *Tetraodon POMCα* Promoter Activity

Bilateral adrenalectomy (ADX) performed in transgenic mice expressing construct 3 showed a robust increase in whole-mount X-Gal staining in pituitary anterior lobe (AL) in comparison with sham-operated siblings (control). No changes were observed in the intermediate lobe (IL). *Left panels* show *insets* of the pituitaries shown on the right. NL, Neural lobe.

Conservation of Hormonal Regulation of Pituitary *POMC* Expression between Fishes and Mice

Adrenal glucocorticoids normally exert a tonic inhibition on *POMC* expression in mouse corticotrophs, whereas hypothalamic CRH activates transcription of

POMC in this cell type. Therefore, we investigated whether corticotropic transgenic expression driven by the *Tetraodon POMC*α promoter was responsive to changes in the concentration of hormones of the mouse HPA axis. We observed that removal of circulating glucocorticoids after bilateral adrenalectomy increased the transcriptional activity of the *Tetraodon POMC*α promoter in corticotrophs of transgenic mice (transgene 3) as evidenced by enhanced X-Gal staining in the pituitary anterior lobe (Fig. 3). The increase in corticotropic expression was dramatic because β-galactosidase-positive cells were undetectable in all of the anterior lobe sections obtained from three sham-operated transgenic mice, whereas three adrenalectomized transgenic mouse siblings showed a high, although variable, number of X-Gal-positive corticotrophs: 137.4 $\pm$ 28.6; 86.8 $\pm$ 31.2, and 79.2 $\pm$ 21.8 per 16-μm coronal pituitary section.

The effect of CRH on *Tetraodon POMC*α promoter activity was directly tested in the mouse corticotropic cell line AtT20 transiently transfected with transgene 3 and compared with the effect of CRH on the mouse *Pomc* promoter (transgene 6; Fig. 1). CRH (10^{-7} M) increased *lacZ* activity driven by the *Tetraodon* or mouse *POMC* promoters to a similar extent (44 and 34%, respectively) in comparison with untreated controls (Fig. 4A). Taken together, these results indicate that *Tetraodon POMC*α promoter activity in mouse corticotrophs is regulated by HPA hormonal changes. In contrast, the glucocorticoid receptor agonist dexamethasone failed to inhibit the activity of the *Tetraodon POMC*α promoter in AtT20 cells as it did acting on the mouse *POMC* proximal promoter (Fig. 4B), suggesting that the mouse ligand-bound glucocorticoid receptor would not interact with *Tetraodon POMC*α promoter sequences. Lack of dexamethasone effect on *Tetraodon POMC*α promoter activity was confirmed in AtT20 cells transfected with transgene 2 (data not shown).

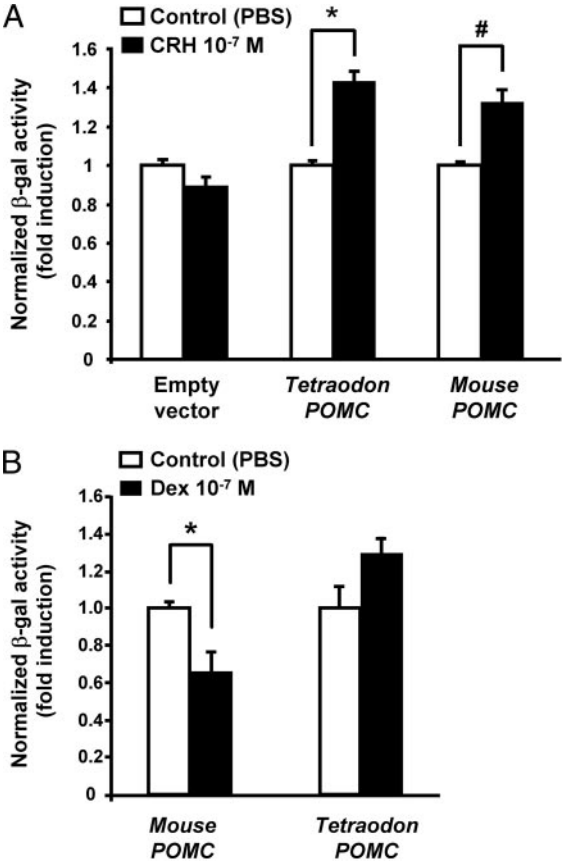


Fig. 4. Effects of CRH and Dexamethasone in *Tetraodon POMC*α Promoter Activity

Mouse corticotropic AtT20 cells were transfected with the empty vector pTrap, transgene 3 (*Tetraodon POMC*), or transgene 6 (mouse *POMC*). A, CRH (10^{-7} M) increased β-galactosidase activity driven by the *Tetraodon POMC*α promoter by 44% with respect to PBS-treated cells, whereas stimulation of the mouse *Pomc* promoter was 33%. B, Dexamethasone (Dex; 10^{-7} M) caused no significant changes in the activity of the *Tetraodon POMC*α promoter and inhibited the mouse *POMC* promoter with respect to PBS-treated cells. Data shown are averages of three to five independent experiments performed in triplicates and expressed relative to PBS-treated controls. *Error bars* represent SEM. *, $P < 0.001$; #, $P < 0.01$, calculated with Student's *t* test.

Phylogenetic Footprinting of the *POMC* Locus at the Vertebrate Extremes: Discovery of a Functional DNA Relic

The results shown above demonstrate that the transcription factor machinery present in mouse pituitary POMC cells specifically recognizes *cis*-acting elements present in the teleost fish *Tetraodon nigroviridis POMC*α promoter. The functional conservation of this *cis-trans* code for more than 450 million years of vertebrate evolution contrasts with the lack of overt sequence similarity between the mouse and *Tetraodon POMC*α 5′-flanking regions (Fig. 5A). The sequence comparison program PipMaker (22), which allows the global visualization of all local alignments, can detect sequence identity between *Tetraodon* and mammals only in *POMC* coding exon 3 (Fig. 5A). To investigate the possibility that only very short functional sequences are conserved among all vertebrates, we used Footprinter 2.1, a bioinformatic program that performs multiple local sequence alignments, taking into account the phylogenetic relationships of the ortholog DNA fragments to be compared, and detects conserved motifs that are evolving more slowly than surrounding DNA sequences (23). By comparing around 700 bp of the *POMC* 5′-flanking sequences of representative species of most vertebrate classes, we were able to define comparison parameters that led to the identification of a short consensus sequence of 10 bp with an identical 4-bp core from human to fishes (Fig. 5, B and C). All species except frogs have this element represented twice in their *POMC* promoter. In mammals, chicken, and zebrafish, these two motifs are separated by around 100 bp, whereas in the Tetraodontid fishes *Tetraodon nigroviridis* and *Takifugu ru-*

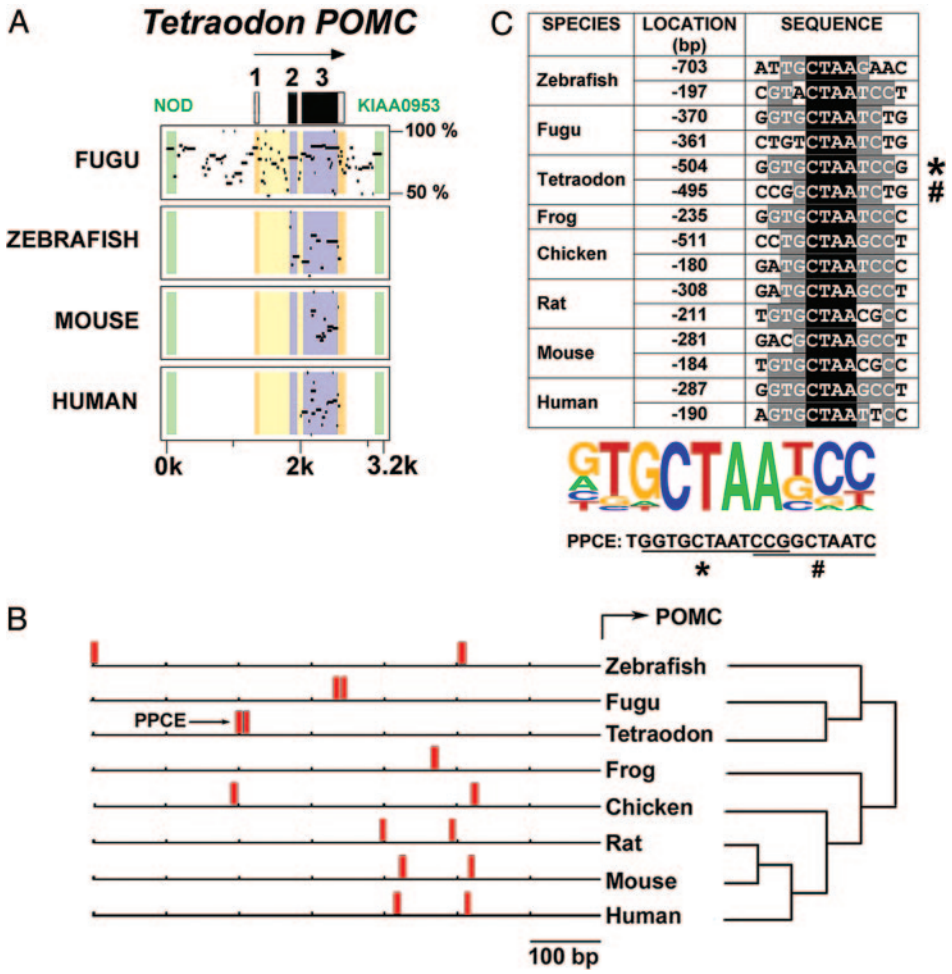
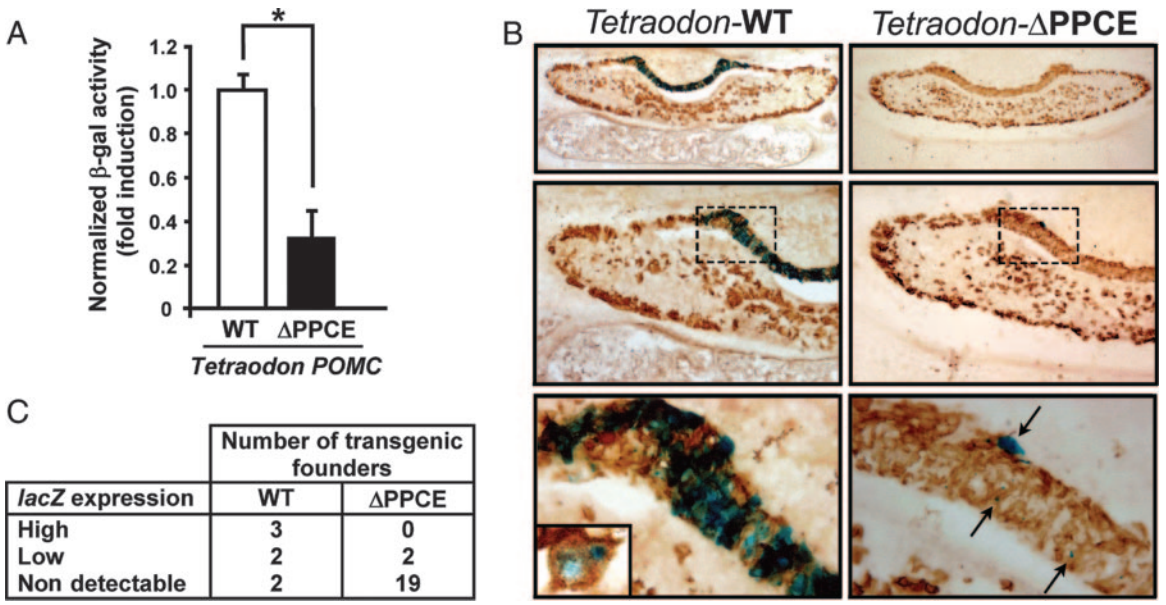


Fig. 5. Phylogenetic Footprinting of Vertebrate *POMC* Loci and Identification of a Conserved 10-bp Motif

A, Percent identity plot using PipMaker for the *Tetraodon POMCα* locus in comparison with its ortholog genes from Fugu, zebrafish, mouse, and human. *Tetraodon POMCα* exons are depicted at the *top*. Coding sequences are depicted in *blue* and untranslated regions in *orange*. Note that *Tetraodon* and mammalian *POMC* show sequence identity only in exon 3. *Tetraodon* and Fugu *POMC* orthologs show a high level of identity across the entire locus. For all species, sequences include the *POMC* gene and approximately 1300 bp or approximately 600 bp of 5′- and 3′-flanking regions, respectively. Part of the upstream and downstream flanking genes (NOD/Caterpiller and KIAA0953, respectively) are denoted in *green*. *Tetraodon* sequence repeats were masked with the RepeatMasker program. B, Output of FootPrinter 2.1 program showing the location of the conserved sequences (*red boxes*) in various vertebrate *POMC* promoters. The conserved sequence present in *Tetraodon* (PPCE) is indicated. *POMC* transcriptional start sites are aligned (*arrow*). Phylogenetic relationships between the species are indicated at the right. Zebrafish, Fugu, and *Tetraodon* sequences correspond to the *POMCα* paralogs. C, Sequence of the conserved motifs identified by Footprinter 2.1. Nucleotides conserved in all sequences are denoted in *black*, and those conserved in most sequences are denoted in *gray*. Nucleotide relative frequencies of the consensus GTGCTAA(T/G)CC are represented below the table. *Tetraodon* PPCE is formed by two juxtaposed conserved motifs (* and #).

bripes, they are next to each other sharing the consensus core sequence GCTAATC (Fig. 5, B and C). We named these juxtaposed elements present in the *Tetraodon POMCα* promoter pituitary POMC conserved element (PPCE).

To investigate whether PPCE plays a role in pituitary *POMC* expression, we performed transient transfection assays in AtT20 cells using a plasmid containing *Tetraodon-POMCα* promoter from which PPCE was deleted (*Tetraodon*-ΔPPCE; transgene 5 in Fig. 1) in comparison with a control plasmid with an intact promoter (*Tetraodon*-WT; transgene 4 in Fig. 1). Removal of PPCE decreased *lacZ* expression by 68% (Fig. 6A). We also tested the importance of *Tetraodon POMCα* PPCE to drive *lacZ* expression to pituitary POMC cells by performing a transgenic mouse founder analysis of postnatal d 1 mice that developed from individual embryos microinjected with transgenes 4 and 5. The intact promoter was able to direct *lacZ* expression to melanotrophs and corticotrophs in five of seven independent transgenic founder mice, whereas removal of PPCE dramatically impaired transcriptional specificity and strength of the *Tetraodon POMCα* promoter (Fig. 6, B and C). From 21 independently generated



C

	Number of transgenic founders	
lacZ expression	WT	ΔPPCE
High	3	0
Low	2	2
Non detectable	2	19

Fig. 6. PPCE Is Critical for *Tetraodon POMCα* Promoter Activity

A, Deletion of PPCE (*Tetraodon*-ΔPPCE, transgene 5) resulted in a 68% fall in β-galactosidase activity in AtT20 cells relative to the intact promoter (*Tetraodon*-WT, transgene 4). β-Galactosidase activity was measured with orthonitrophenyl 1-β-D-galactopyranoside, and EGFP was used as transfection efficiency control. pTrap (empty vector) background expression was subtracted from both WT and ΔPPCE activity. B, Coronal pituitary sections of newborn (postnatal d 1) *Tetraodon*-WT (*left panels*) or *Tetraodon*-ΔPPCE (*right panels*) transgenic founder mice showing colocalization of β-galactosidase (X-Gal staining, *blue dots*) and ACTH immunoreactivity (*brown*) in melanotrophs and corticotrophs (only visible at higher magnification). Objective magnifications: *upper panels*, ×5; *middle panels*, ×10; *lower panels*, ×40. The *dashed rectangles* represent the magnified figures below. The *inset* in the *left corner* shows a corticotroph positively stained with X-Gal (×100). The *arrows* show some of the scarce X-Gal-positive melanotrophs in ΔPPCE transgenic mice. C, Number of transgenic founders showing high expression (*e.g. left panels* of B), low expression (*e.g. right panels* of B), and nondetectable expression of *lacZ* in pituitaries of WT and ΔPPCE mice.

Tetraodon-ΔPPCE transgenic founder mice, only two showed X-Gal staining in just a few POMC cells, whereas the other 19 mice were completely devoid of pituitaty *lacZ* expression (Fig. 6, B and C). From these latter 19 mice at least 10 showed ectopic *lacZ* expression in different brain areas, indicating that absence of pituitary expression was not due to insertion of the transgene in silent heterochromatin. Together, these results demonstrate that the 20-bp DNA element PPCE present in Tetraodontid *POMCα* promoters plays a critical role in the *cis-trans* code controlling pituitary *POMC* expression in transgenic mice.

Although critical for basal corticotropic expression, PPCE is dispensable for CRH stimulation of *POMC* expression because CRH (10^{-7} M) increased *lacZ* activity in AtT20 cells transfected with the wild-type or the PPCE mutant plasmid in a similar fashion (88 *vs.* 53%, respectively; Fig. 7A).

In the human, mouse, rat, and chicken *POMC* promoters, the upstream conserved 10-bp relic is similar to a previously characterized element present in the rat *POMC* promoter that acts as a binding site for Pitx1 (10, 24). Because Pitx1 has been reported to stimulate rat *POMC* promoter activity, we investigated whether Pitx1 would also regulate *Tetraodon POMCα* promoter activity interacting with PPCE. To this end, we performed a functional expression study in heterologous Chinese hamster ovarian (CHO) cells transfected with either *Tetraodon*-WT (construct 4), *Tetraodon*-ΔPPCE (construct 5), or the mouse proximal *Pomc* promoter (construct 6). Cotransfection with a plasmid expressing mouse Pitx1 increased the activity of the wild-type and the mutated construct to a similar extent (Fig. 7B), indicating that Pitx1 stimulation of *Tetraodon POMCα* is independent of PPCE and that this element might be specifically recognized by a still unknown transcription factor present in pituitary *POMC* cells.

Tpit is a T box transcription factor known to play an important role in pituitary *POMC* expression in humans and mice (13). To study whether Tpit also enhances *Tetraodon POMCα* promoter activity, we cotransfected CHO cells with the same reporter plasmids as in the previous experiment together with a plasmid expressing Tpit. Figure 7C shows that Tpit was able to stimulate *Tetraodon POMC* in a similar fashion to the mouse ortholog and through a PPCE-independent mechanism.

Tetraodon PPCE Acts as a Transcriptional Enhancer in the Mouse *Pomc* Promoter

Because PPCE proved to be crucial for *Tetraodon POMCα* promoter activity, we then investigated whether this fish element would also act as a pituitary

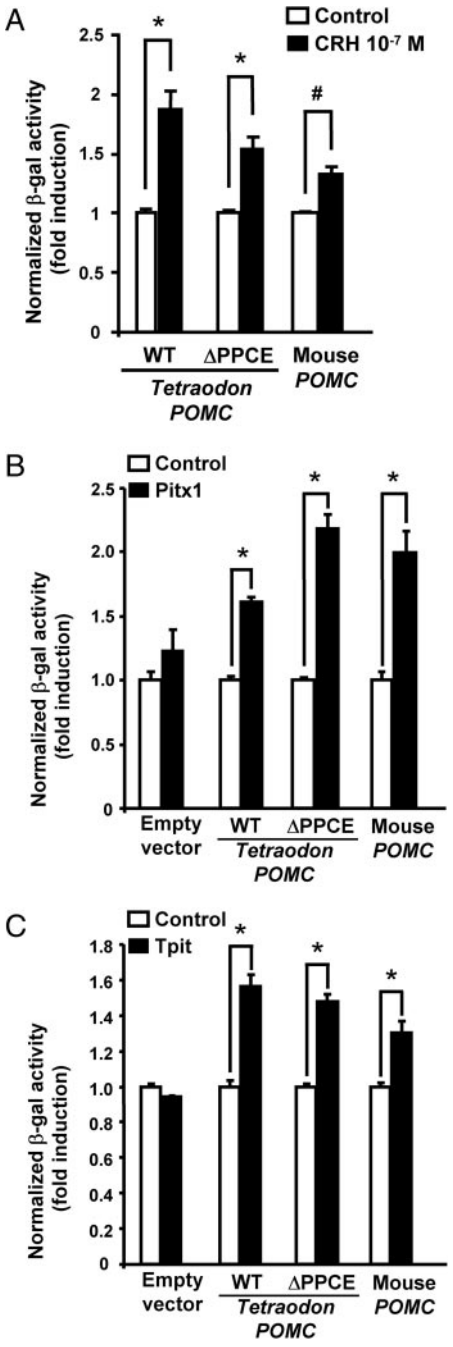


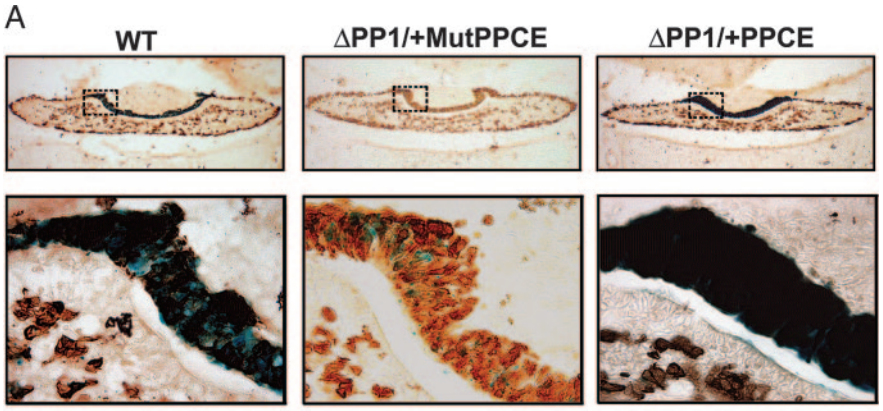
Fig. 7. CRH, Pitx1, and Tpit Stimulation of *Tetraodon POMC*α Promoter Activity Does Not Depend on PPCE

A, AtT20 cells were transfected with the empty vector pTrap, transgene 4 (*Tetraodon*-WT), transgene 5 (*Tetraodon*-ΔPPCE), or transgene 6 (mouse POMC). Treatment with 10^{-7} M CRH increased the activity of *Tetraodon POMC*α promoter independently of PPCE. B, CHO cells were transfected with the same reporter plasmids as in A together with a mouse Pitx1 expression vector (pcDNA3-Pitx1) or empty pcDNA3 as control. C, Similar experiment as in B, but cotransfection was performed with Tpit (pcDNA3-Tbx19). Data are expressed relative to PBS-treated (A) or pcDNA3 cotransfected (B and C) controls. *Bars* are the mean of three independent experiments performed in triplicates. *Error bars* represent SEM. *, $P < 0.001$; #, $P < 0.01$, calculated with Student's *t* test.

POMC cell enhancer when placed into the mouse *Pomc* promoter context. The mouse and rat *POMC* promoters have two discrete 10-bp motifs separated by approximately 100 nucleotides that are highly similar to PPCE sequences. Our laboratories previously identified the more proximal of these motifs [named putative pituitary POMC-1 (PP1)], relative to the TATAA box, and showed by a deletion analysis performed in transgenic mice that it plays an important pituitary enhancer role (9, 25). We therefore swapped PP1 from the mouse *Pomc* promoter with PPCE (transgene 7) or with a mutated PPCE sequence (transgene 8) and performed a second transgenic newborn mouse founder study. Consistent with our previously published studies based on the rat *POMC* promoter (9, 25), transgene 6 showed strong expression of β-galactosidase in pituitary POMC cells (Fig. 8, A and B). The exchange of PP1 for a mutated version of PPCE considerably diminished the transcriptional strength of the mouse *Pomc* promoter as evidenced by fewer and less intense X-Gal-stained pituitary POMC cells. In contrast, replacement of PP1 by intact fish PPCE restored, and even increased, *Pomc*-specific expression levels in pituitary melanotrophs and corticotrophs (Fig. 8, A and B). Interestingly, the percentage of transgenic mouse founders expressing *lacZ* in POMC cells was also greatly improved in the presence of PPCE (Fig. 8B). In general, an increased penetrance of transgene expression from multiple independent integration sites is highly suggestive of increased enhancer function overcoming negative position effects. The forceful evidence of this experimental sequence swap indicates that the conserved 10-bp element detected by Footprinter 2.1 in proximal *POMC* promoters is a functionally conserved relic that putatively controls pituitary *POMC*-specific expression in all vertebrate species.

DISCUSSION

The impending completion of several whole-genome projects has greatly stimulated comparative studies that attempt to understand how genomes were shaped by evolution. The theory elaborated by Kimura and Takahata (26) several decades ago establishing that the rate of molecular evolution is inversely related to the level of functional constraint can also be demonstrated now for noncoding sequences and, in particular, those that participate in transcriptional regulation (27). Although enhancers evolve more slowly than neighboring sequences, their identification by phylogenetic footprinting is not always applicable because transcription factor binding sites are short, accept a certain degree of degeneracy, and may disappear and be recreated in a different region at a relatively fast turnover rate (28, 29). In fact, absence of conservation in flanking sequences of ortholog mammalian and fish genes prevails (30). In this study, we sought to inves-



B

	Number of transgenic founders		
lacZ expression	WT	ΔPP1/+MutPPCE	ΔPP1/+PPCE
High	2	0	3
Low	0	1	3
1-3 positive cells	1	1	0
Non detectable	8	5	3

Fig. 8. *Tetraodon* PPCE Enhances Mouse *Pomc* Promoter Activity in POMC Cells of Transgenic Mice

The PP1 binding site in the mouse *Pomc* promoter was deleted and replaced with either a wild-type *Tetraodon* PPCE or a mutated PPCE. A, Pituitary coronal sections of newborn (postnatal d1) founder mice carrying transgene 6 (WT, *left panels*), transgene 8 (ΔPP1/+MutPPCE, *middle panels*), or transgene 7 (ΔPP1/+PPCE, *right panels*) showing β-galactosidase (X-Gal staining, *blue dots*) and ACTH immunoreactivity (*brown*). Objective magnifications: *upper panels*, ×5; *lower panels*, ×40. The *dashed rectangles* represent the magnified figures below. B, Number of transgenic mice showing high expression (*e.g. left* and *right panels* of A), low expression (*e.g. middle panels* of A), or nondetectable expression of reporter *lacZ* in pituitaries of WT, ΔPP1/+MutPPCE, and ΔPP1/+PPCE mice.

tigate whether the loss of overall sequence conservation between two ortholog promoters of extremely distant species modifies the molecular interaction between *cis*-acting elements and *trans*-acting factors. To this end, we generated transgenic mice in which *lacZ* pituitary expression was driven by the 5′-flanking sequence of the *POMCα* gene of the teleost fish *Tetraodon nigroviridis*. In *Tetraodon, POMCα* is expressed in the intermediate (pars intermedia) and anterior (pars distalis) lobes of the pituitary (8), a similar expression pattern to that of the mammalian *POMC* gene. Our results demonstrated that, in the absence of overt sequence similarity, mammalian and fish species share the *cis*-element information necessary for specific gene expression in pituitary melanotrophs and corticotrophs. Thus, the regulatory elements of *POMCα* that are functional in mammalian pituitary cells are probably also at work in the *Tetraodon* pituitary. These results are in line with a recent study showing that enhancers of the human *RET* locus, conserved only among mammals, drive specific transgene expression in zebrafish, even though there is no obvious sequence conservation between human and fish noncoding ortholog sequences (31). Taken together, we conclude that ortholog enhancers decrease their sequence conservation in direct relation to their evolutionary distance even when preserving their function. Exceptions to this rule are 100- to 500-bp ultraconserved enhancers particularly found in developmental genes (32).

As a vital part of the HPA axis, one of the critical properties of *POMC* promoter activity is that it is stimulated by CRH and inhibited by glucocorticoids. In our transgenic mice, glucocorticoid depletion induced by bilateral adrenalectomy strongly stimulated reporter gene activity driven by the *Tetraodon POMCα* promoter, suggesting that the molecular mechanisms that participate in glucocorticoid regulation of *POMC* expression are conserved at the vertebrate extremes. The increase in *Tetraodon POMCα* promoter activity observed in adrenalectomized mice could be due to the lack of direct glucocorticoid repression on the fish *POMC* promoter and/or increased stimulation of hypothalamic CRH release. Our experiments performed in the mouse corticotropic cell line AtT20 showed that CRH increased *Tetraodon POMCα* promoter activity, but dexamethasone did not reduce basal promoter activity as it did on the mouse ortholog promoter. Thus, although glucocorticoid-mediated repression of *POMC* transcription appears to be different between mammals and teleost fishes, the final regulatory effect of glucocorticoid depletion on corticotropic *POMC* promoter activity is conserved.

Altogether these observations raise a fundamental question in molecular evolution: how do preserved mechanisms of precise gene expression control coexist with an absence of sequence conservation? One possible explanation is that the conserved expression phenotype observed between the mouse and *Tetraodon POMC* promoters is the result of a stabilizing

selection process, in which multiple near-neutral compensatory mutations occurred since the divergence from a common ancestor almost half a billion years ago. This type of mechanism has already been demonstrated at the molecular and functional level for two species of *Drosophila* whose most recent common ancestor diverged around 50 million years ago (33). A nonexclusive alternative explanation is that ortholog pituitary *POMC* enhancers evolved at a neutral rate except for a reduced number of very short sequences that maintained their efficacy to bind ortholog transcription factors to assure pituitary-specific *POMC* expression from mammals to fishes. In this study, we investigated the possibility that very short elements are conserved in *POMC* promoters from a wide spectrum of vertebrate species, including teleost fishes, amphibians, birds, and mammals. By using the program FootPrinter 2.1 (23), we identified a 10-bp conserved motif that is present twice in all ortholog *POMC* promoters except in *Xenopus tropicalis*, where it is present just once. In *Tetraodon*, both motifs are juxtaposed (PPCE), and when deleted, transcriptional activity of the *Tetraodon POMC*α promoter markedly decreased in both the mouse corticotropic cell line AtT20 and pituitary POMC cells of transgenic mice. Interestingly, the mammalian conserved motifs correspond to reported binding sites for pituitary-specific nuclear proteins. The upstream motif is recognized by Pitx1, a homeodomain transcription factor that is expressed during the development of the pituitary and participates in *POMC* expression (10, 24). However, deletion of PPCE did not modify the ability of Pitx1 to increase *Tetraodon POMC*α promoter activity. In addition, the downstream element recognized by the FootPrinter program in mammalian promoters is known to bind to a protein present in AtT20 nuclear extracts, and there is strong evidence that this element is necessary for rat *POMC* promoter activity in the pituitary of transgenic mice (9, 25). The putative transcription factor that binds to this element, dubbed PP1, has not yet been identified and might be a factor that regulates the expression of *POMC* in both teleosts and mammals. In fact, whereas swapping PP1 from the mouse promoter for a mutated version of fish PPCE impaired transcriptional activity of the chimeric promoter, its exchange for intact PPCE completely restored reporter gene activity in pituitary POMC cells of transgenic mice, demonstrating that the mouse and fish elements are functional analogs. Although the functional 10-bp relic was the only strongly conserved sequence found using this analysis, pituitary *POMC* expression in mammals is known to depend on the synergistic activity of several distinct binding sites and transcription factors (34). Comparisons between more closely related species might be necessary to identify additional functional elements. *Tetraodon*, similarly to all other teleost fishes, has two *POMC* paralogs that likely originated in the whole-genome duplication that occurred in a teleost ancestor around 320 million years ago (8). Expression of the *Tetraodon POMC* paralogs has diverged during evolution; *POMC*α is expressed strongly in the pituitary and in the ventral hypothalamus, whereas *POMC*β is expressed weakly in the pituitary and in the preoptic area in the brain. Consistent with the divergence of their expression patterns, the 5′-flanking region of teleost *POMC*β genes does not have the consensus short element present in teleost *POMC*α and the *POMC* promoter of all other vertebrates.

Even though the pituitary regulatory regions are functionally conserved between the *POMC* promoters of fishes and mammals, transgenic mice generated with the entire *Tetraodon POMC*α locus failed to express the transgene in the eight pedigrees analyzed. Although some tetraodontid genomic clones have been accurately expressed in transgenic mice (35), there are other examples in which they have failed to be properly expressed, probably due to a fish-mouse incompatibility on mRNA processing or minimal promoter recognition (20, 21). Our results provide a clear example that the study of teleost regulatory regions in mammalian expression systems can be maximized by using fusion transgenes containing heterologous minimal promoters and classical reporter genes such as *lacZ*.

In summary, we have found that pituitary cell-specific and hormonally regulated expression of *POMC* from a teleost fish is functionally conserved in mammalian expression systems despite great promoter sequence divergence. A bioinformatic analysis that takes into account the evolutionary distance of all compared species allowed the identification of a unique 10-bp motif that is conserved in all vertebrate classes and proved to be essential for pituitary *POMC* transcriptional regulation. Future experiments will be needed to determine whether transcription factors that control *POMC* expression in mammals also control the teleost orthologs, shedding light on the molecular evolution of the *cis-trans* transcriptional code of this essential gene.

MATERIALS AND METHODS

Tetraodon Genomic Library Generation and Screening

A fosmid library was constructed with the genomic DNA of *Tetraodon nigroviridis* using the EpiFOS Fosmid Library Production Kit (Epicenter, Madison, WI). Using a 968-bp PCR product of the *POMC*α promoter ($-1124/-157$) as probe, we screened the genomic DNA library spotted onto nylon membranes and found two positive clones (044-N21 and 057-A19).

Transgenes and Plasmids

Transgenes were constructed using standard molecular cloning techniques, and sequence quality was assessed by automated PCR sequencing. To obtain transgene 1, a 3578-bp *NarI*/*Xho*I restriction fragment was isolated from the 044-N21 clone containing the complete *POMC*α locus and part of both 5′- and 3′-flanking genes and subcloned into pBluescript SK

(+/−) (Stratagene, La Jolla, CA). To obtain transgene 2, transgene 1 was digested with *Hin*dIII, producing a fragment of 1723 bp (−1328/+395) that includes part of the upstream gene, the 5′-flanking region, the first noncoding exon, and part of the first intron of *Tetraodon POMCα*. This fragment was subcloned into the pTrap vector (36), upstream of the minimal promoter of the chicken β-globin gene and the *lacZ* reporter gene. To obtain transgene 3, a 967-bp fragment of the 5′-flanking region of *POMCα* (−1124/−157) was PCR-amplified using *Tetraodon* genomic DNA and the primers 5′-GGG*GTCGAC*NGGTTCTNCTTTGCCTTG-3′ (*Sal*I restriction site *underlined*) and 5′-GGG*GGTACC*TCATTCATGCT-NGTAAACAC-3′ (*Kpn*I site *underlined*), and the PCR product was subcloned into the pTrap vector into the *Sal*I/*Kpn*I sites. Before microinjection, transgene 1 was digested with *Eco*RV and *Xho*I, and transgenes 2 and 3 were digested with *Not*I to discard vector sequences. Digested plasmids were size-fractionated on agarose gels and purified using Elutip columns (Schleicher and Schuell, Keene, NH).

To obtain transgene 5, a 20-bp sequence containing PPCE (TGGTGCTAATCCGGCTAATC) was deleted using a mega-primer PCR approach (37). Two external primers were used to amplify a 1064-bp fragment (−1193/−130) of *Tetraodon POMCα* 5′-flanking region: primer 1, 5′-CCC*GTCGAC*AGAA-CACAGGCTGGTTT-3′ (*Sal*I site *underlined*), and primer 2, 5′-CCC*AAGCTT*GTCACAGGTGATGTA-3′ (*Hin*dIII site *underlined*). For the deletion of PPCE, two internal primers were designed, each containing both 5′ and 3′ PPCE flanking sequences: primer 1b, 5′-TGTCAGCGGGCAGGCCGCCAT-GGCGTCATG-3′, and primer 2b, 5′-GCCATGGCGGCCTGC-CCGCTGACATTTAG-3′. First, two parallel PCR were performed using primers 1/1b (5′ fragment amplification) and 2/2b (3′ fragment amplification) using transgene 1 as template. Both PCR products are complementary in the PPCE flanking regions and therefore were used as template and primers (megaprimers) for a second PCR, together with external primers 1 and 2. This second reaction generated a product lacking PPCE. To obtain the wild-type control (transgene 4), a PCR amplification was performed using primers 1 and 2. The final products were cut with *Sal*I and *Hin*dIII and subcloned into the pTrap vector. To obtain transgene 6, a 472-bp fragment (−503 to −31) of the mouse *Pomc* promoter was amplified by PCR using the primers 5′-CCC*GTC-GAC*CCGTGACTCTTG-3′ (*Sal*I site *underlined*) and 5′-CCC*AAGCTT*CTTGCAGGGTTG-3′ (*Hin*dIII site *underlined*). The product was subcloned into the *Sal*I/*Hin*dIII sites of the pTrap vector. To construct transgenes 7 and 8, the PP1 site was replaced from transgene 6 by PPCE or by a mutated PPCE (TGGTG*TCGG*TCCGG*TCGG*TC, mutations *underlined*). The external primers for both constructs are 5′-CCC*GTCGAC*CCGTGACTCTTGACAGCCTCTGTTGTT-3′ (*Sal*I site *underlined*) and 5′-CCC*AAGCTT*CTTGCAGGGTT-GGGTGGGTGAGCCTTGG ACGTG-3′ (*Hin*dIII site *underlined*). The internal primers to swap PPCE were 5′-*GA-TTAGCCGGATTAGCACCA*ACCCGCTGAAGCGCA-3′ and 5′-*TGGTGCTAATCCGGCTAATC*GCCAGCCTCCGCGCTTT-3′ (PPCE *underlined*). To swap the mutated PPCE, the internal primers were 5′-*GACCGACCGGACCGACACCA*CCCGCT-GAAGCGCA-3′ and 5′-*TGGTGTCGGTCCGGTCGGTC*GC-CAGCCTCCGCGCTTT-3′ (mutated PPCE *underlined*). PCR products were subcloned into the *Sal*I/*Hin*dIII sites of the pTrap vector. pcDNA3-Pitx1 and pcDNA3-Tbx19, expression plasmids for mouse Pitx1 and Tpit/Tbx19, respectively, were kindly provided by M. G. Rosenfeld (38).

Transgenic Mice and Adrenalectomy

Transgenic mice were generated by pronuclear microinjection of B6CBF2 or B6D2F2 zygotes as described previously (17, 39). Microinjected zygotes were transferred to the oviduct of B6CB pseudopregnant females. Transgenic pups were identified by tail genomic DNA PCR as described (17, 39) using the primer pair 5′-CGGTTCTGCTTTGCCTTG-3′ and 5′-TCATTCATGCTTGTAAACAC-3′, which amplifies the *Tetraodon POMCα* promoter. Bilateral adrenalectomy was performed as described previously (39), and mice were analyzed 4 d after adrenalectomy for X-Gal staining. Sham-operated control mice were subjected to the same surgery, but adrenal glands were left intact. Experimental protocols were consistent with the Guide for the Care and Use of Laboratory Animals and approved by the local institutional animal care and use committee.

Histochemistry

For X-Gal staining, adrenalectomized or sham-operated adult mice were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in KPBS (0.9% NaCl, 16 mM K_2HPO_4, 3.6 mM KH_2PO_4). Pituitaries were excised, postfixed in 4% PFA/KPBS for 15 min at room temperature, and incubated overnight with 1 mg/ml X-Gal (Invitrogen, Carlsbad, CA) in staining solution (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4; 2.12 mg/ml potassium ferrocyanide; 1.64 mg/ml potassium ferricyanide; 2 mM $MgCl_2$; 0.01% sodium deoxycholate; and 0.02% Nonidet P-40, pH 7.5) at 37 C. The number of X-Gal-positive cells in the anterior lobe was quantified in seven to 12 coronal half-cryosections of 16 μm thickness (three mice per group). For β-galactosidase and ACTH colocalization, postnatal d 1 transgenic mice carrying constructs 2, 4, 5, 6, 7, or 8 were decapitated, and skin-free heads were fixed with 4% PFA/KPBS for 1 h at 4 C and subjected overnight to whole-mount X-Gal staining. Stained heads were cryoprotected with 30% sucrose/KPBS and frozen in embedding medium (Tissue Tek; Electron Microscopy Sciences, Hatfield, PA). Serial coronal sections (20 μm) were cut using a cryostat microtome (IEC Microtome, Walldorf, Germany) at −20 C. Sections were thaw-mounted on Vectabond-coated (Vector Laboratories, Burlingame, CA) slides and stored at −70 C until use. X-Gal-stained slices were then subjected to anti-ACTH immunohistochemistry as described in de Souza *et al.* (17) except that the incubation with anti-ACTH antibody was conducted at 37 C for 4 h and slices were developed with 25 mg/ml diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.05% H_2O_2 in Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, pH 7.5). The *in situ* hybridization of brains and pituitaries of transgene-1 mice was performed as described in de Souza *et al.* (17) with the probe for *Tetraodon POMCα* exon 2 described in de Souza *et al.* (8).

Cell Culture and Transient Transfection Assays

AtT20 and CHO cells were cultured in DMEM supplemented with 10% fetal calf serum. For dexamethasone treatment, charcoal-stripped fetal calf serum was used. For lipofection, 90% confluent attached cells were incubated with DNA and Lipofectamine 2000 (Invitrogen) as suggested by the manufacturer. For CRH and dexamethasone treatment, one well of a six-well dish was transfected with each plasmid. The next day, cells were passed to 96-well plates and incubated either with 10^{-7} M CRH (National Hormone and Pituitary Program, National Institutes of Health, Torrance, CA), 10^{-7} M dexamethasone (Decadron, Sidus, Argentina) or PBS for 24 h. CHO cells were transfected in 96-well plates with 130 ng/well of transgene 4 or 5 and 20 ng/well of pcDNA3-Pitx1, pcDNA3-Tbx19, or pcDNA3 (Invitrogen) as control. Fifteen nanograms of CMV-EGFP plasmid per well were used for transfection efficiency control. pcDNA3 was used to complete a total of 200 ng DNA/well. β-Galactosidase activity was measured with orthonitrophenyl 1-β-D-galactopyranoside (MP Biomedicals, Solon, OH) and normalized with protein (Bradford; Bio-Rad, Hercules, CA) or by counting EGFP-positive cells.

Sequences and Programs

POMC loci sequences from human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*), zebrafish (*Danio rerio*), *Tetraodon* (*Tetraodon nigroviridis*), and Fugu (*Takifugu rubripes*) were retrieved from the Ensembl website (www.ensembl.org). The 700-bp sequences of *POMC* promoters of the different vertebrate species were compared using the FootPrinter 2.1 program (23) available at http://bio.cs.washington.edu/software.html. We fixed parameters for searching 7-bp sequences conserved in all the species with a maximum of two mutations per sequence and one mutation per branch. Sequences of *POMC* loci of *Tetraodon*, Fugu, zebrafish, mouse, and human were compared using the PipMaker program (http://bio.cse.psu.edu/pipmaker) after masking the repeats with RepeatMasker program (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker). Nucleotide frequencies of the conserved elements were calculated using the WebLogo program available at http://weblogo.berkeley.edu/.

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