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Molecular cloning, sequence analysis and expression of the snake follicle-stimulating hormone receptor

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Abstract

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) control gonadal function in mammalian and many non-mammalian vertebrates through the interaction with their receptors, FSHR and LHR. Although the same is true for some reptilian species, in Squamata (lizards and snakes) there is no definitive evidence for the presence of either two distinct gonadotropins or two distinct gonadotropin receptors. Our aim was to characterize the gonadotropin receptor(s) of the *Bothrops jararaca* snake. Using a cDNA library from snake testis and amplification of the 5′-cDNA ending, we cloned a cDNA related to FSHR. Attempts to clone a cDNA more closely related to LHR were unsuccessful. Expression of FSHR mRNA was restricted to gonadal tissues. The snake FSHR is a G protein-coupled receptor with 673 amino acids, and the aminoterminal domain with 346 amino acids consists of a nine leucine-rich repeat-containing subdomain (LRR) flanked by two cysteine-rich subdomains. The β-strands in the LRR are conserved with exception of the third, a region that may be important for FSH binding. In contrast with mammalian, avian and amphibian FSHRs, the snake FSHR presents amino acid deletions in the carboxyterminal region of the extracellular domain which are also seen in fish and lizard FSHRs. cAMP assays with the recombinant protein transiently expressed in HEK-293 cells showed that the snake FSHR is more sensitive to human FSH (hFSH) than to human chorionic gonadotropin. Phylogenetic analysis indicated that the squamate FSHRs group separately from mammalian FSHRs. Our data are consistent with the apparently unique gonadotropin-receptor system in Squamata reptilian subgroup. Knowledge about the snake FSHR structure may help identify structural determinants for receptor function.

Keywords: Gonadotropin receptors; FSH and LH receptor; Cloning; Snake; *Bothrops jararaca*

1. Introduction

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) control the gonadal function in mammals and many non-mammalian vertebrates. These hormones act on specific receptors in the cell membrane, respectively, the FSHR and the LHR. These receptors belong to a subfamily of the G protein-coupled receptors (GPCRs) superfamily, now called leucine-rich repeat-containing GPCRs (LGRs), which also includes the TSH receptor and the more recently described sea anemone LGR (Nothacker and Grimmelikhuijzen, 1993), snail LGR (Tensen et al., 1994), fruit fly LGR1 and LGR2 (Hauser et al., 1997; Nishi et al., 2000), nematode LGR (Kudo et al., 2000) and human LGR4 to 8 (Hsu et al., 1998, 2000, 2002). All members of this subfamily contain a large extracellular aminoterminal domain consisting of three subdomains: an N-terminal cysteine-rich subdomain followed by a nine leucine-rich repeat-containing subdomain (LRR subdomain) and a C-terminal cysteine-rich subdomain. The leucine-rich repeats in the LRR subdomain have been postulated to form a horseshoe-shape important for hormone binding (Jiang et al., 1995).

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Classical biochemical studies identified two gonadotropins in many non-mammalian vertebrates, including amphibia, reptiles, birds (Licht et al., 1977; Farmer et al., 1975) and fish (Suzuki et al., 1988a,b). More recently, molecular studies confirmed the presence of two gonadotropins in many non-mammalian vertebrates. cDNAs encoding the α and β chains of fish gonadotropins (Kitahara et al., 1988; Sekine et al., 1989), avian LH and FSH (Ando and Ishi, 1994; Shen and Yu, 2002), amphibian LH and FSH (Saito et al., 2002), and turtle LH and FSH (Aizawa and Ishii, 2003) have been cloned.

Conversely, both biochemical and molecular biology studies failed to identify two gonadotropin hormones in the Squamata (snakes and lizards). Classical biochemical studies identified a gonadotropin hormone in snakes with intermediate characteristics between LH and FSH (Licht et al., 1977, 1979). Molecular biology studies identified only a cDNA encoding the lizard LH but not FSH β chain (Aizawa and Ishii, 2003). However indirect evidence from Southern blot and immunohistochemistry studies suggests the existence of two distinct gonadotropins in Squamata (Borrelli et al., 1997; Desantis et al., 1998).

The gonadotropin receptors LHR and FSHR were first described in mammals (McFarland et al., 1989; Sprengel et al., 1990), but cDNAs encoding two distinct receptors have also been described for birds (Johnson et al., 1996; Mizutani et al., 1998; You et al., 1996) and fish (Oba et al., 1999a,b). In amphibians and reptiles, however, only cDNAs encoding FSHRs have been described so far (Borrelli et al., 2001; Nakayama et al., 2000). Special effort in studying these two groups may help clarify the molecular evolution of gonadotropin system in vertebrates.

Their high evolutionary rate compared to the other reptiles makes the Squamata an interesting group for study (Olmo et al., 2002). In addition, the remarkable flexibility in their reproductive strategies makes the snakes a particularly important group for reproduction studies (Shine, 2003). Nevertheless to date there are no reports on the gonadotropin-receptor system in any snake.

Thus, our aim was to identify the gonadotropin receptors in a snake. We here present the first molecular cloning, expression and phylogenetic analysis of a snake gonadotropin receptor that is closely related to FSHRs.

2. Materials and methods

2.1. Animals

Male and female *Bothrops jararaca* (Serpentes, *Crotalinae*) snakes were captured from the wild and classified by the Butantan Institute, Laboratory of Herpetology (São Paulo, Brazil). All procedures were approved by the Committee for Bioethics in Animal Experimentation of the Butantan Institute. Snakes were decapitated and tissues and blood were removed to obtain genomic DNA or total RNA.

2.2. cDNA library construction and screening

Total RNA from snake testes was obtained with the TRIzol reagent (Gibco-BRL, Rockville, MD) according to the standard protocol (Chomczynski and Sacchi, 1987). The mRNA was isolated with the RNA Message Maker kit (Gibco). The cDNA library was constructed using the ZAP cDNA Gigapack cloning kit (Stratagene, La Jolla, CA).

To obtain a probe to screen the library for LHR or FSHR-related sequences, we performed a polymerase chain reaction (PCR), using the library as a template and two degenerate primers (1 and 2; Table 1) derived from regions with high homology among the vertebrate glycoprotein hormone receptors LHR, FSHR, and TSHR (Hauser et al., 1997). These primers were designed against the aminoterminal region of the first transmembrane domain and the second transmembrane region. The reaction mixture consisted of 10^7 plaque forming units of the cDNA library, 2.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, and 2.5 U *Taq* Polymerase (Gibco), in 50 μL total volume. The PCR consisted of an initial denaturation step of 94 °C for 5 min, and 35 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 2 min, with an extension complement of 72 °C for 7 min. The PCR products were resolved on a 1.5% agarose gel and the product with the expected size (158 bp) was subcloned in the pGEM-T-easy vector (Promega, Madison, WI) for automated sequencing (ABI Prism Big Dye Terminator kit, Applied Biosystems, Foster City, CA). The 158 bp product was labeled with ^{32}P (7T Quick Prime kit, Amersham–Pharmacia Biotech, Piscataway, NJ) and used as a probe to screen the cDNA library, according to standard methodology (Maniatis et al., 1982). Screening of the cDNA clones

Table 1
Sequences of the primers used

Primer number	Sequence (5′ to 3′)
1	CCITGYGARGAYATIATGGITA
2	GCRAAGCMSAGRTTRCACATIAGRAA
3	RTGTRCTCCAGAACCAGATGCYTTYA
4	GAGCAGCARGTARAGCCCCA
5	RTRGCCTTCSTYRTCATYTG
6	ARGARTTGACWGGRTARAAW
7	AACCAGCCCCAGTTTGCCAGTCAATAGC
8	CTCGAGTCAAGAAGGCATGGGCAACGAAGA
9	GGATCATGATTTGATAACCTTTGAC
10	CCGCTCGAGAAGCATGGCTCTGTTTTTC
11	GGATCATGATTTGATAACCTTTGAC

*Xho*I sites are underlined.

was performed after transfer of the lysis plaques to nitrocellulose or Hybond NX nylon membranes (Amersham).

As mentioned before, the pair of primers described above should amplify both FSHR and LHR-related sequences. To amplify only LHR-related sequences we used two other sets of primers (sense 3 and antisense 2 or 4; Table 1). These primers should amplify a region between the aminoterminal of the first transmembrane domain and the second transmembrane domain (nucleotide positions 1026–1211 and 1026–1242 of the human LHR). A third set of degenerate primers (5 and 6; Table 1) designed to amplify a region between nucleotides 1747 and 1968 of the cDNA sequence of the chicken LHR (Mizutani et al., 1998) was also used. The templates for PCR were the testes cDNA library, genomic DNA or cDNA from ovaries collected in January and August. Chicken genomic DNA was used as a control.

2.3. Amplification of the 5′-cDNA end (5′-RACE)

The 5′ end of the cDNA was obtained with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). The total RNA and the mRNA from snake testis were prepared as described above. The first cDNA strand for the 5′-RACE was obtained according to the instructions of the manufacturer. The gene specific primer 7 (Table 1) was derived from the sequence of the clone obtained in the screening of the library. The PCR conditions were: 5 cycles of 94 °C for 30 s, 72 °C for 2 min; 5 cycles of 94 °C for 30 s, 70 °C for 30 s and 72 °C for 2 min; 27 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 2 min. The main PCR product was subcloned in the PGEM-T easy vector (Promega) and sequenced.

2.4. Amplification of the whole open reading frame for the snake gonadotropin receptor

The complete cDNA coding for the snake gonadotropin receptor was obtained by PCR overlap extension using the product obtained from the cDNA library (a cDNA insert in pBluescript, linearized with *Xho*I) and the cDNA obtained from the RACE (subcloned in pGEM T-easy and linearized with *Xba*I) as templates, and the sense primer 8 and antisense 9 (Table 1). The PCR conditions were: 2 μL of each template, 25 pmol each primer, 0.2 mM dNTP and 2.5 U Platinum *Taq* high fidelity polymerase (Gibco) in 50 μL total volume, using the following parameters: 1 cycle of 94 °C for 4 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min; and 1 cycle of 72 °C for 7 min. The product was subcloned in pGEM-T easy vector and submitted to three independent sequencing analyses of the sense and antisense strands.

2.5. Sequence analysis

The identity of the sequence with other glycoprotein hormone receptors was analysed with BLAST (Altschul et al., 1997). The signal peptide cleavage site was predicted at the Signal PWorld Wide Webserver (http://www.cbs.dtu.dk/services/SignalP-2.0/). The seven membrane-spanning regions were predicted at the server HMMTOP (http://www.enzim.hu/hmmtop). Prediction of the Ser, Thr, and Tyr phosphorylation sites was done at http://www.cbs.dtu.dk/services/NetPhos/. The potential N-glycosylation sites were predicted at www.cbs. dtu.dk/services/NetNGlyc/. To analyse the conservation of the extracellular, transmembrane and carboxyterminal regions of the receptors shown in Fig. 2, the aminoacid sequences were aligned by ClustalW v. 1.8 (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) and displayed using the Boxshade 3.21 software (http:searchlauncher.bcm.tmc.edu/multi-align/multi-align.html).

2.6. Phylogenetic analysis

We compared the sequences from 18 taxa, including FSHR, LHR, and TSH receptor (TSHR) from *Homo sapiens* (GenBank Accession Nos. M65085, NM000233, and M32215, respectively), *Bos taurus* (L22319, AF491303, and U15570), FSHR and LHR from *Rattus norvegicus* (L02842 and M26199), *Gallus gallus* (U51097 and AB009283), *Oncorhynchus rhodurus* (AB030012 and AB030005), FSHRs from *Podarcis sicula* (AJ292553), *Bothrops jararaca* (present study, AY189696), and *Cynops pyrrhogaster* (AB005587), TSHR from *Ovis aries* (Y13434), and leucine-rich repeats-containing GPCRs (LGRs) from the invertebrates *Drosophila melanogaster* (U47005) and *Anthopleura elegantissima* (Z28332).

The nucleotide sequences were assembled and aligned using the Se-Al package (v. 2.0, Rambaut, 2002). Nine hundred and fifty four sites were analysed. All three codons positions were included in the data set for the tree reconstruction. Sites containing gaps were treated as new state (fifth base). The tree reconstruction was performed by means of the Neighbor-Joining (NJ) algorithm with uncorrected distance. Missing-ambiguous data were distributed proportionally to unambiguous changes, all substitutions were estimated under Minimum Evolution model. We also employed the branch-and-bound algorithm under Maximum Parsimony (MP) criteria, using PAUP* v. 4.0b10 software (Swofford, 2002). For the character state optimization, we used ACCTRAN, and the swapping algorithm was TBR. Unrooted trees were performed. Our analysis also included the LGR sequences from sea anemone and *Drosophila* in order to examine branch length among clades. Support for nodes in the reconstructions was obtained by bootstrapping the data sets in PAUP*, with

500 replicates and full heuristic search. Consistency and Retention index (CI and RI, respectively) were obtained using MacClade v. 4.0 software (Maddison and Maddison, 2000).

2.7. Construction of plasmids for expression in mammalian cells

The coding region of the snake gonadotropin receptor cDNA was amplified by PCR, using the high fidelity Vent polymerase (New England Biolabs, Beverly, MA) with the sense primer 10 and antisense primer 11 (Table 1). The PCR product was digested with *Xho*I and subcloned in pcDNA 3 (Invitrogen) for expression in mammalian cells.

To visualize the expression of the receptor protein in HEK 293 cells using comercially available antibodies, we used PCR strategies to insert a FLAG tag (DYKDD DDK) immediately after the sequence encoding the signal peptide. Thus, the mature protein contained the tag at its aminoterminal end. All plasmids were sequenced three independent times to check the fidelity of the PCR.

2.8. Cell culture and transient expression in human embryonic kidney HEK-293 cells

HEK-293 cells were grown in Ham's F12 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and 50 μg/mL gentamycin at 37 °C. For transfection experiments, cells were plated in gelatin-coated 100 mm dishes at a density of 3×10^5 cells/plate, and transfected two days later with 20 μg empty vector or the snake gonadotropin receptor, using the calcium phosphate precipitation method (Chen and Okayama, 1987). Cells were used two days after transfection for RNA extraction and Northern blot experiments. For the cyclic AMP assays, cells were trypsinized one day after transfection, plated on 96-well plates and used one day later.

2.9. Northern blot assays

Total RNA was extracted from tissues (gonads, heart, kidney, and liver) freshly removed from adult *B. jararaca* snakes or from HEK-293 cells transiently transfected with empty vector or with the snake gonadotropin receptor. Five micrograms of total RNA was applied to the agarose/Mops gel. The gel-separated RNAs were transferred for 2 h to Hybond membranes (Amersham) using the Posiblot system (Stratagene). UV cross-linked membranes were prehybridized for 3 h in 6× SSC (standard saline citrate)/5× Denhardt's solution, 0.5% SDS, and 100 μg/mL sheared denatured salmon sperm DNA at 65 °C. Hybridization was performed overnight at 65 °C in the same solution containing the ^{32}P-labeled 158 bp PCR product corresponding to the nucleotides from position 1003 to 1160 of the snake FSHR cDNA. The membranes were then washed for 15 min three times with 2× SSC/0.1% SDS at room temperature, and twice with 1× SSC/0.1% SDS at 65 °C. Membranes were exposed to film for autoradiography during one week to measure endogenous expression and 24 h to measure expression of the recombinant receptor in HEK 293 cells.

2.10. Fluorescence microscopy

Cells were plated on coverslips in six-well plates (2×10^5 cells/well). They were transfected with 4 μg of empty vector or with the FLAG-tagged snake gonadotropin receptor. Two days after transfection, cells were fixed with 3.7% formaldehyde for 30 min at 37 °C and washed three times with PBS. Cells were blocked with phosphate-buffered saline (PBS) containing bovine serum albumin for 30 min at room temperature, and washed three times in PBS. The samples were incubated for 2 h at room temperature with 2.5 μg/mL mouse anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MI). After washes, cells were incubated for 30 min with 10 μg/mL rabbit anti-mouse Ig G conjugated to rhodamine and then for 30 min with goat anti-rabbit IgG (Alexafluor 594 Signal amplification kit, Molecular Probes, Eugene, OR). The receptor was visualized with a fluorescence microscope (E800, Nikon). The images were captured and analysed through the Image-Pro Express software (Media Cybernetics, Silver Spring, MD).

2.11. Total cAMP assay

Human FSH (hFSH) and human chorionic gonadotropin (hCG) were provided by Dr. Parlow and National Hormone and Peptide Program (NHPP, Torrance, CA).

Total cAMP in transfected cells was measured by enzyme immunoassay (Biotrak System, Amersham–Pharmacia Biotech). After a 30 min pre-incubation period in 160 μL serum-free medium containing 0.5 mM isobuthyl-methylxanthine, cells were incubated for 30 min at 37 °C with 20 μL PBS (controls) or 0.1, 1.0, or 10 μg/mL of hFSH or hCG. After the incubation period, cells were lysed and aliquots were transferred to the assay plate, according to the instructions of the manufacturer. A standard curve of cAMP (12.5–3200 fmol/well) was obtained and the values were read from this curve. Results are expressed as a percentage of the value obtained for the non-stimulated controls (cells transfected with the plasmids that were incubated with PBS instead of the agonists). To check the reliability of the method cells transfected with empty vector were stimulated with 10^{-5} M forskolin for 5 min.

The effect of different concentrations of the agonist was compared by one-way ANOVA, followed by the Newman–Keuls test. The level of significance was $P < 0.05$.

3. Results

3.1. Molecular cloning of the snake gonadotropin receptors

Using a cDNA library from snake testis and degenerate oligonucleotides derived from regions conserved among vertebrate glycoprotein hormone receptors, we amplified a 158 bp product that was used as a probe to screen the library. Using nitrocellulose membranes we screened at least 2×10^6 plaques, and obtained only one positive clone. Using Hybond nylon membranes several positive clones were obtained but they were all unrelated to gonadotropin receptors. After in vivo excision and sequencing, searches in databases showed that the positive clone encoded the serpentine domains and the carboxyterminal region of a putative gonadotropin receptor. Using a gene specific primer derived from this sequence in a 5′-RACE reaction we obtained the whole aminoterminal region plus the first and second transmembrane domains. The region of complete overlap between the clone from the library and the product of the 5′-RACE was 262 bp long.

The 2100 bp cDNA sequence containing the complete open reading frame with 2019 bp was obtained by PCR overlap extension and is shown in Fig. 1. The predicted protein with 673 aa is a putative G protein-coupled receptor with seven transmembrane spanning regions and a large aminoterminal region containing 346 aa, including the 17 aa of the predicted signal peptide. The putative start codon is preceded by an in-frame ATG that starts at position −21 (Fig. 1). Although both ATG codons are flanked by consensus sequences for the initiation of translation (Kozak, 1987), comparisons with receptors from other species suggest that the second ATG is used as the start codon. Regardless of which ATG is used as the start codon, cleavage of the signal peptide leads to the same mature protein with 656 amino acids.

3.2. Sequence analysis of the snake gonadotropin receptor

Analysis in the Blast database revealed that the cloned snake cDNA sequence has a high identity with FSHRs from other species (Table 2). Identity of the new receptor with vertebrate LH and TSH receptors is much lower (about 55 and 31%, respectively). The cloned receptor presents a high aminoacid identity with FSHRs of the chicken and the lizard (72 and 71%, respectively) and slightly less with the FSHRs of the newt and man (69 and 67%, respectively).

```
                                                    TCAAGAAGGCATGGGCAACGAAGAAGCAAGC   -1
ATGGCTCTGTTTTTCCTCAGCCTGTGGCTCCTCTTGGGGGCTTGCTTAGGGTGCCAGCAT    60
 M  A  L  F  F  L  S  L  W  L  L  L  G  A  C  L  G ↑C  Q  H     20
CCCCTCTGTCAGTGCTCGGACAGAATGGCATTCATCTGCCAGGAGAGTGAAGTGACCTAT   120
 P  L  C  Q  C  S  D  R  M  A  F  I  C  Q  E  S  E  V  T  Y     40
GTACCACAGGACATCCCCAGGAATTCCACTGAACTGATATTTTTCCTCACCAAAATAAGA   180
 V  P  Q  D  I  P  R  N  S  T  E  L  I  F  F  L  T  K  I  R     60
ATCATCCCCAAAGGAGCTTTCTTGGGATTTGGAGAAGTGGAAAAAATAGAGATCTCTCAA   240
 I  I  P  K  G  A  F  L  G  F  G  E  V  E  K  I  E  I  S  Q     80
AATGATGCCCTGGAAACTATAGAATCAGATGTTTTCTCACACCTGCCCAAGCTTTATGAA   300
 N  D  A  L  E  T  I  E  S  D  V  F  S  H  L  P  K  L  Y  E    100
ATGATTGAGAAAGCAAACAATCTTGTGTATATTGATAGAAATGCATTCCAGAAACTTCCC   360
 M  I  E  K  A  N  N  L  V  Y  I  D  R  N  A  F  Q  K  L  P    120
AGTCTCAGATATTTGTTAATATCGAACACTGCCATCCGATTTTTGCCTGTTGTTAACCAA   420
 S  L  R  Y  L  L  I  S  N  T  A  I  R  F  L  P  V  V  N  Q    140
GTATACGCTTTGCAGAAAGTTGTGTTAGATATTCAAGACAACATAAATATTCGCAAAATT   480
 V  Y  A  L  Q  K  V  V  L  D  I  Q  D  N  I  N  I  R  K  I    160
GAAAGAAATTCTTTTCTTGGCCTAAGTTCTGATCGTGTTGATATAAGACTGGATAAAAAT   540
 E  R  N  S  F  L  G  L  S  S  D  R  V  D  I  R  L  D  K  N    180
GGAATACCTGAAATTGAAAACCATGCCTTCAATGGGACTATCTTGTCTGAGCTAAATCTG   600
 G  I  P  E  I  E  N  H  A  F  N  G  T  I  L  S  E  L  N  L    200
AGTGATAATTATAATCTAGAAAAGTTACCTAATGAAGTTTTCAAAGGAGCACATGGACCA   660
 S  D  N  Y  N  L  E  K  L  P  N  E  V  F  K  G  A  H  G  P    220
GGTTATTTGGATATTTCTGAGACAAAGATCAGCCAACTACCAAGCATTGGTCTAGAGCAC   720
 G  Y  L  D  I  S  E  T  K  I  S  Q  L  P  S  I  G  L  E  H    240
ATTAACAAGTTGGTAGCAAAATCTACATACAATTTAAAAAAGCTCCCTCCCTTGGACAAG   780
 I  N  K  L  V  A  K  S  T  Y  N  L  K  K  L  P  P  L  D  K    260
TTCCATGCTTTGATTGAGGCAAACCTCACATATCCAAGCCACTGCTGTGCATTTGAAAAT   840
 F  H  A  L  I  E  A  N  L  T  Y  P  S  H  C  C  A  F  E  N    280
CGGACTAAACAAAATTCTGTGATGCACCCTATATGCAATAAATCTGCAATTTCAGAAGAG   900
 R  T  K  Q  N  S  V  M  H  P  I  C  N  K  S  A  I  S  E  E    300
TCTGATGAGTTCAGTTTTGAGCTGGATGAAAATGATTACTATCATGCTCTATGTAGAGAA   960
 S  D  E  F  S  F  E  L  D  E  N  D  Y  Y  H  A  L  C  R  E    320
GAGTTTAAAGTGGCTTGCTTCCCAGAGCCTGATGCCTTCAACCCATGTGAAGACATAATG  1020
 E  F  K  V  A  C  F  P  E  P  D  A  F  N  P  C  E  D  I  M    340
GGCCACATAATTCTTCGAGTCCTGATATGGTTCATCAATATCTTGGCCATCATGGGGAAT  1080
 G  H  I  I  L  R  V  L  I  W  F  I  N  I  L  A  I  M  G  N    360
CTTATTGTGTTCATCATTTTAATCAGCAGCCAATACAAGCTCACTGTCCCTCGATTCCTT  1140
 L  I  V  F  I  I  L  I  S  S  Q  Y  K  L  T  V  P  R  F  L    380
ATGTGCAATCTTGCATTTGCTGATCTTTGCACAGGTATTTATTTACTACTGATTGCCATC  1200
 M  C  N  L  A  F  A  D  L  C  T  G  I  Y  L  L  I  A  I      400
AAAGACATGCAGAGTAGAACTCAGTATTATAACTATGCTATTGACTGGCAAACTGGGGCT  1260
 K  D  M  Q  S  R  T  Q  Y  Y  N  Y  A  I  D  W  Q  T  G  A    420
GGTTGCAATACAGCTGGTTTTTTCACTGTATTTGCAAGTGAACTCTCCGTATACACTTTA  1320
 G  C  N  T  A  G  F  F  T  V  F  A  S  E  L  S  V  Y  T  L    440
ACCGTCATCACATTAGAACGATGGCACACCATCACCTATGCCATGGAACTAGACCGCAAA  1380
 T  V  I  T  L  E  R  W  H  T  I  T  Y  A  M  E  L  D  R  K    460
GTCCGCTTCCGTCATGCAGTGATAATTATGCTGGTAGGATGGGTCTTTGCCTTTACAGTG  1440
 V  R  F  R  H  A  V  I  I  M  L  V  G  W  V  F  A  F  T  V    480
GCCCTCCTTCCCATATTTGAAGTCAGCAGCTACATGAAAGTTAGCATCTGCCTACCTATG  1500
 A  L  L  P  I  F  E  V  S  S  Y  M  K  V  S  I  C  L  P  M    500
GATATTGAAACTCTGCTTGCCCAGACCTACGTCATGTTTTTGCTGATATTAAATATTCTC  1560
 D  I  E  T  L  L  A  Q  T  Y  V  M  F  L  L  I  L  N  I  L    520
GCCTTTGTTATCATCTGTTCCTGCTACATCAGCATCTACTTCACTGTGAGGAATCCCAAT  1620
 A  F  V  I  I  C  S  C  Y  I  S  I  Y  F  T  V  R  N  P  N    540
GTTTTCTCATCCAACAGTGACACCAAGATTGCCAAACGCATGGCCATACTGATCTTCACA  1680
 V  F  S  S  N  S  D  T  K  I  A  K  R  M  A  I  L  I  F  T    560
GATTTCCTTTGCATGGCACCAATCTCCTTTTTTGCCATTTCAGCATCACTCAAGGTCCCT  1740
 D  F  L  C  M  A  P  I  S  F  F  A  I  S  A  S  L  K  V  P    580
CTGATCACTGTGTCCAATTCAAAGATCCTCCTTGTTTTGTTCTACCCCATTAATTCATGT  1800
 L  I  T  V  S  N  S  K  I  L  L  V  L  F  Y  P  I  N  S  C    600
GCCAATCCATTTCTTTATGCCATCTTCACGAAGACATTTCGCAGGGATTTTTTCATCCTA  1860
 A  N  P  F  L  Y  A  I  F  T  K  T  F  R  R  D  F  F  I  L    620
CTAAGTAAGTTTGGCTGCTGTGAAATGTATGCTCAGATTTACAGAACGGAAACCTCCTCT  1920
 L  S  K  F  G  C  C  E  M  Y  A  Q  I  Y  R  T  E  T  S  S    640
TCTGTTCACACCTCCCATATGAAAAATGGGCATTGTACACCAGCTTCTAAAACCAGTGAT  1980
 S  V  H  T  S  H  M  K  N  G  H  C  T  P  A  S  K  T  S  D    660
GGAACAATTTATTCATTAGTGCCTCTGAATCATGTGAACTGAAGCAGAATGGCTGTAAAT  2040
 G  T  I  Y  S  L  V  P  L  N  H  V  N  *                      673
TTATGTCAAAGGTTATCAAATCATGATCC                                  2069
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Fig. 1. The cDNA and predicted amino acid sequence of the snake FSHR. Nucleotides (upper line) are numbered from the 5′- to 3′-end, beginning with the putative start codon. An asterisk indicates the translation termination codon. The predicted signal cleavage site is indicated by an arrow. The putative seven transmembrane domains are boxed. Single underlines indicate potential N-glycosylation sites. Double underlines indicate protein Kinase C Consensus Phosphorylation sites. A dashed line indicates potential GRKz Consensus Phosphorylation sites.

Table 2 also shows that the transmembrane region of the receptor is especially well conserved. The most variable region among species was the aminoterminal exodomain. In this region the snake receptor had a relatively high identity with the FSHR of the lizard.

Table 2
Amino acid identity (%) of the snake FSHR with FSHRs of other species

Species	Overall	Receptor region		
		Aminoterminal	Transmembrane	Carboxyterminal
Gallus gallus	72	59	86	82
Podarcis sicula	71	65	81	76
Cynops pyrrhogaster	69	58	82	70
Homo sapiens	67	59	78	66
Rattus norvegicus	67	59	77	64

Aminoterminal region. In common with the other members of the glycoprotein hormone receptor subfamily the snake FSHR presents a large extracellular region (346 amino acids, including the 17-residue signal peptide). In this region, six potential N-linked glycosylation sites are found at positions 48, 191, 199, 268, 280, and 293 (Fig. 1).

The aminoterminal exodomain of the snake FSHR has an LRR subdomain with nine leucine-rich repeats, flanked by two cysteine-rich subdomains with 10 cysteine residues (Fig. 2). The four cysteines in the N-terminal cysteine-rich subdomain are also present in human, chicken, and newt FSHRs, but in the lizard FSHR the first and the third conserved cysteines are absent. The six conserved cysteines in the C-terminal cysteine-rich subdomain are present in all species analysed (Fig. 2).

The LRRs usually start with β-strands composed of a highly conserved $X^1X^2LX^3LX^4X^5$ motif, in which X

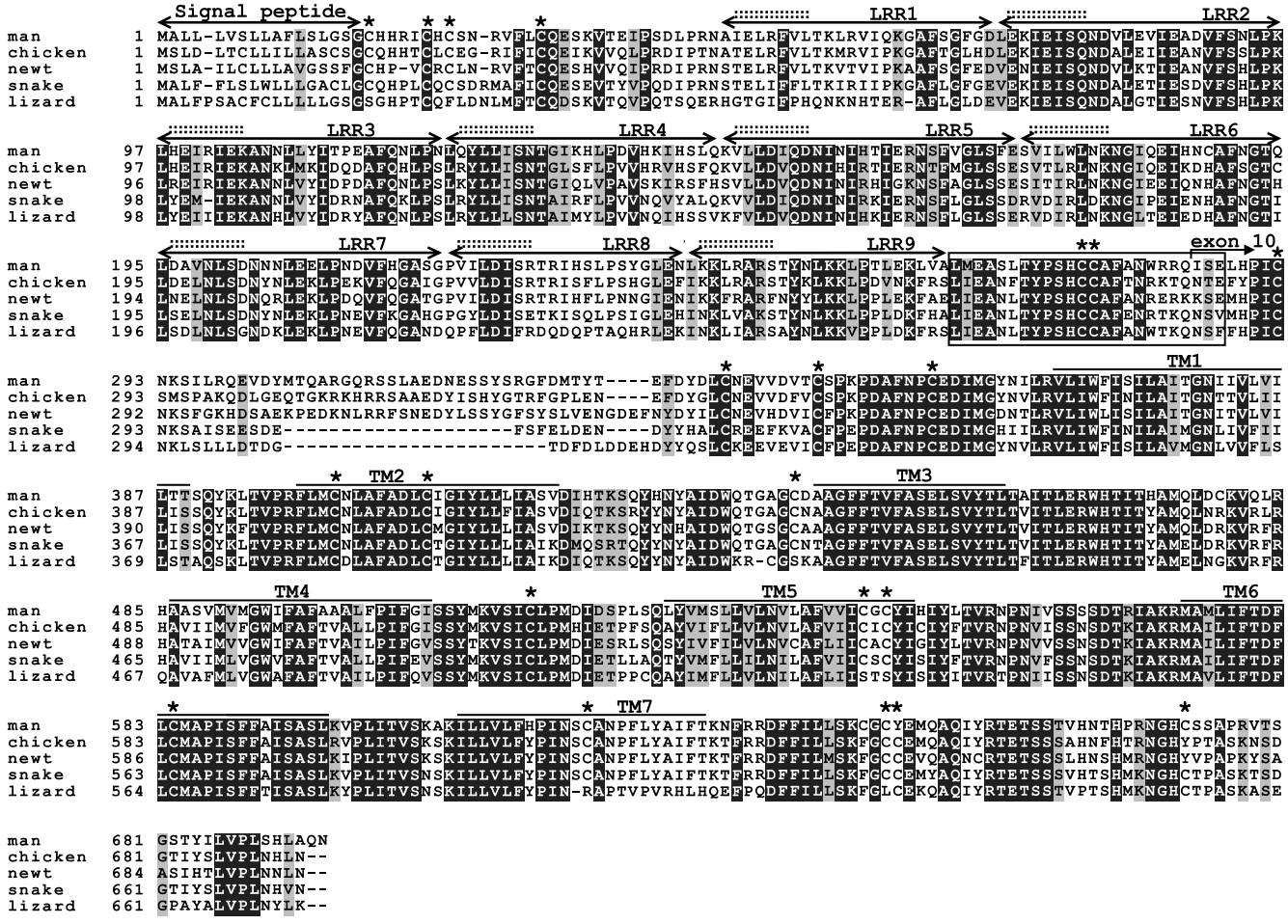

Fig. 2. Alignment of the snake FSHR with FSHRs from several species. The ectodomain of FSHRs is encoded by nine exons, and consists of a signal peptide to direct the protein to the cell membrane, the LRR1-9 subdomains, demarcated with arrows, and two cysteine-rich subdomains flanking the LRRs. Dashed double lines indicate the β-strands in each LRR. The C-terminal cysteine-rich subdomain contains the hinge region that is boxed. The conserved cysteine residues are marked with asterisks. Region encoded by exon 10 is indicated by an arrow. The seven transmembrane domains (TM) are also indicated. Spaces were introduced to increase alignment. Numbers at the left refer to the first amino acid position. Consensus amino acid residues are shaded. The darkest shaded residues represent a complete conservation of the five receptors used for comparison.

indicates any amino acid and *L* refers to leucine, isoleucine or other hydrophobic residues (Kobe and Kajava, 2001). Interestingly, the snake receptor does not have the conserved β-strand in LRR3 (Fig. 2). In addition, the LRR3 of the snake FSHR comprises 24 amino acids, one less than the other species analysed.

In common with FSHRs of other species, the snake FSHR presents the conserved hinge region after the LRR9. Remarkably, both the snake and the lizard FSHRs present deletions not shared with mammalian, avian or amphibian FSHRs (Fig. 2).

Transmembrane and carboxyterminal regions. Two highly conserved cysteines that can form a disulphide bridge are present in the first and in the second extracellular loops of the snake FSHR. There is one potential serine phosphorylation site in the third intracellular loop at position 544 and 9 potential phosphorylation sites (S640, S641, S645, T612, T638, T653, T658, Y630, and Y664) in the carboxyterminal region.

3.3. Attempts to clone the snake LHR

We were not able to find cDNAs more closely related to LHR in the *B. jararaca* snake, although we have used different sets of primers and different templates, including genomic DNA, cDNAs from testis and from ovary in different phases of the reproductive cycle, and the cDNA library from snake testis (not shown). Nevertheless, we successfully amplified an LHR fragment from the chicken genomic DNA.

3.4. Phylogeny of the snake FSHR

To investigate the phylogenetic relationship of the snake FSHR with other members of the LGR family, we performed a phylogenetic analysis using MP and NJ methods (Fig. 3A and B, respectively). In the MP analysis, after 3474 rearrangements tried, one tree was retained (Fig. 3A).This topology presented 2664 steps, with CI of 0.502 and RI of 0.500. These support indexes suggest that the data present a homoplastic profile. On the other hand, in both tree topologies the high bootstrap values indicate the presence of three main clades, FSHR, LHR, and TSHR lineages. The TSHR clade presented a bootstrap value of 100 in NJ and MP criteria; LHR, 98 in both criteria; and FSHR, 99 and 100 bootstrap values in NJ and MP, respectively. Inside the FSHR clade we found two very well-supported sister groups: the mammalian clade (bootstrap = 100) and the squamate clade (bootstrap = 78, NJ and 96, MP).

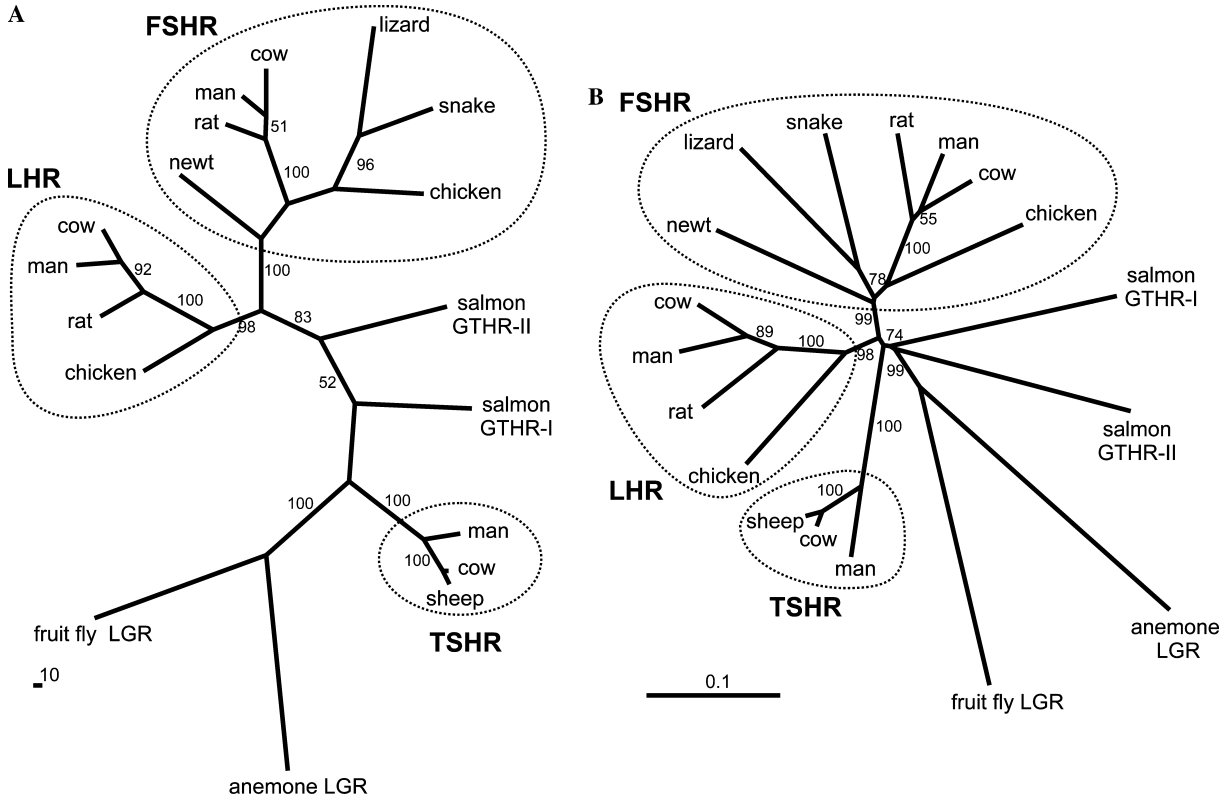

Fig. 3. Phylogenetic trees reconstructed with Maximum Parsimony (A) and Neighbor-Joining (B) methods of the nucleotide sequences of various members of the glycoprotein hormone receptor family. GTHR-I and GTHR-II refer, respectively, to the FSHR and LHR from the salmon (Oba et al., 1999a,b). The bootstrap values are reported on the tree branches, and the three very well-supported clades FSHR, LHR, and TSHR are encircled.

3.5. Endogenous expression of the snake FSHR

Northern blot assays using various tissues of the *B. jararaca* snake revealed a main FSHR specific transcript of about 2.5 kb in ovary and testis, but not in kidney, heart or liver (Fig. 4). A less prominent transcript of about 3.0 kb was also observed in testis and ovary.

3.6. Expression of the recombinant snake FSHR in mammalian cells

Northern blot analysis revealed the presence of the expected 2.5 kb transcript in cells transfected with the snake FSHR but not in those transfected with the empty vector (Fig. 5A). In addition, HEK 293 cells also produced a transcript of about 4.5 kb.

To analyse the expression of the corresponding protein, a FLAG-tagged construct of the snake FSHR was transiently expressed in HEK 293 cells. Immunofluorescence microscopy of non-permeabilized cells revealed labelling of membranes of cells transfected with the snake receptor, but not of those transfected with empty vector (Fig. 5B).

3.7. Functional analysis of the recombinant snake FSHR in mammalian cells

cAMP production after stimulation with forskolin or with increasing concentrations of hFSH or hCG was measured in HEK-293 cells transiently transfected with empty vector or with the coding region of the snake FSHR. hFSH or hCG did not produce any significant increase in cAMP production in cells transfected with empty vector (Fig. 6). Treatment of the same cells with 10^{-5} M forskolin for 5 min increased cAMP production to about 800% of the basal (not shown). hFSH

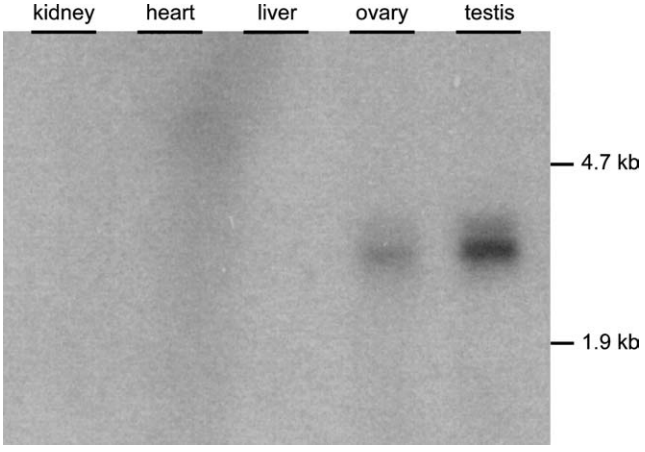

Fig. 4. Expression of FHSR in snake tissues. For Northern blot analysis, 5 μg total RNA extracted from different tissues of adult snakes were probed with a 158 bp fragment of the snake FSHR cDNA. The positions of mammalian ribosomal RNAs are indicated on the right side of the panel. The membranes were exposed to film for 1 week.

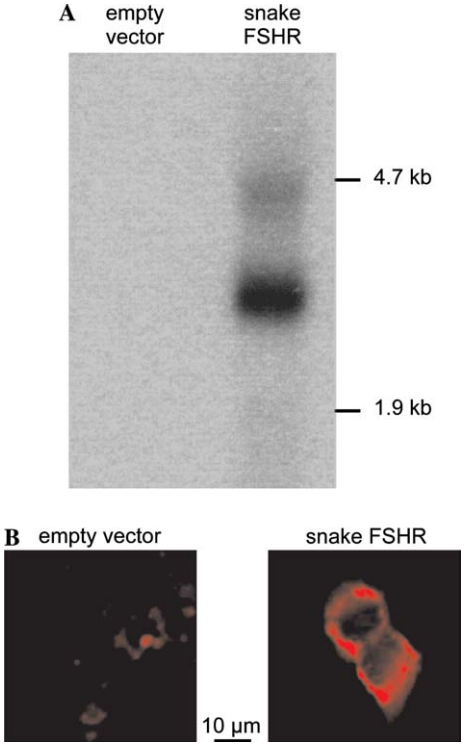

Fig. 5. Expression of the snake FSHR mRNA (A) and the protein (B) in HEK-293 cells. (A) Northern blot analysis: 5 μg total RNA extracted from cells transiently transfected with empty vector or the snake FSHR, were probed with a 158 bp fragment of the snake FSHR cDNA. The positions of mammalian ribosomal RNAs are indicated on the right side of the panel. The membranes were exposed to film for 1 day. (B) Immunofluorescence analysis: cells were transiently transfected with the empty vector or the FLAG-tagged snake FSHR. Non-permeabilized cells were incubated with the monoclonal mouse anti-FLAG M2 antibody (2.5 μg/mL) and a rhodamine-conjugated secondary antibody.

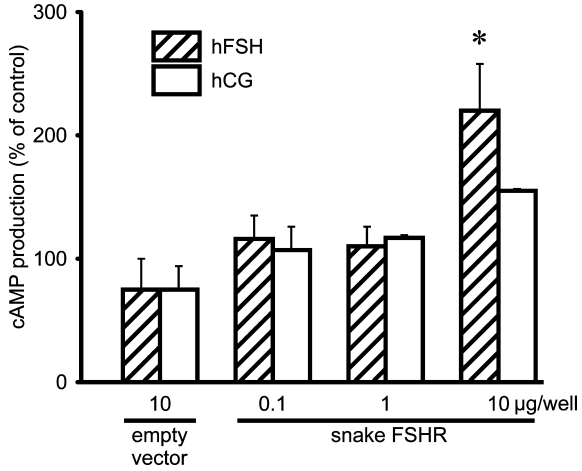

Fig. 6. Cyclic AMP production in HEK-293 cells transiently transfected with empty vector or with the snake FSHR. About 5×10^4 cells/well were stimulated with increasing concentrations of the hFSH or hCG. Total cAMP was determined by EIA. Results are expressed as the percentage of control (transfected cells incubated with buffer instead of hormone). Values are means ± SEM from 3 to 5 independent transfections. An asterisk indicates statistically different from the other doses (ANOVA followed by Newman–Keuls test, $P < 0.05$).

(10 μg/well) significantly increased the cAMP production in cells transfected with the snake FSHR (ANOVA and Newman–Keuls, $P < 0.05$). In contrast, even high doses of hCG failed to stimulate cAMP production in those cells.

4. Discussion

The main purpose of this work was the identification of the gonadotropin receptors from snakes. Using a combination of two techniques, screening of a cDNA library of the *B. jararaca* testis and 5′-RACE, we cloned for the first time a putative gonadotropin receptor with a higher identity with FSHR than LHR of other species.

Despite the high identity with other FSHRs, we identified several peculiar structural features of the snake FSHR that may influence its function. Three main differences were found in the aminoterminal exodomain, which is thought to be responsible for the selectivity and high affinity binding of the corresponding hormone (Ji and Ji, 1991; Tsai-Morris et al., 1990; Xie et al., 1990). First, the snake FSHR contains six potential N-glycosylation sites in the aminoterminal region compared to four sites found in the human FSHR. Three of those sites are conserved in both receptors: N191, N199, and N293. Glycosylation is probably related to proper folding and trafficking of the receptor protein to the membrane.

Second, as in mammalian species, the aminoterminal exodomain of the snake FSHR consists of three subdomains: an N-terminal cysteine-rich subdomain, a nine LRR subdomain, and a C-terminal cysteine-rich subdomain. However, the LRR3 in the snake FSHR does not have the conserved $X^1X^2LX^3LX^4X^5$ motif (Fig. 2). Conversely, the lizard FSHR does not have the conserved hydrophobic residues in the β-strand of LRR1. Whether the change in LRR3 accounts for the poor discrimination between FSH and LH in binding studies with snake tissues (Licht et al., 1977) is not clear. Using chimeric receptors, Moyle et al. (1994) concluded that LRR3 and the N-terminal cysteine-rich subdomain, together with LRR7 and the C-terminal cysteine-rich subdomain, are important for the FSH binding and specificity, but other authors found that most of the β-strand substitutions do not affect responsiveness to FSH (Vischer et al., 2003).

The third peculiar feature of the snake FSHR is the presence of deletions in the carboxyterminal region of the aminoterminal domain. A similar deletion is also seen in the lizard (Fig. 2), whereas a smaller deletion is found in the gecko FSHR (Akazome et al., 1996), and a larger deletion in the fish GTHR-I (Bogerd et al., 2001; Laan et al., 2002). These deletions in the snake FSHR are probably not due to alternative splicing since their location in the human FSHR gene is slightly away from the 5′ border of exon 10 (Fig. 2). In mammalian FSHRs, this region is probably not involved in hormone binding. However, the immediately preceeding hinge region, well conserved in the three glycoprotein hormone receptors, is thought to play a role in conformational changes that follow receptor activation, allowing interaction with the transmembrane region and signal transduction (Nakabayashi et al., 2000; Nishi et al., 2002). It remains to be investigated whether deletions found in Squamata FSHRs could affect the conformation of the activated receptor.

We identified nine potential phosphorylation sites in the carboxyterminal region of the snake FSHR, compared to 4 and 5, respectively, of the rat and the human FSHRs. FSHRs of both man and rat have three potential phosphorylation sites in the third intracellular loop and the snake FSHR has only one. Activation of the gonadotropin receptors leads to their phosphorylation, which affects uncoupling of receptor from the G-protein, and receptor internalization (Lazari et al., 1998, 1999). The intracellular loops were characterized as the region of phosphorylation in the rat FSHR (Nakamura et al., 1998). Therefore, it seems likely that the mechanism of regulation of the snake receptor will differ from that of the mammalian FSHRs.

In summary, there are several interesting structural features in the snake FSHR and we are currently using mutant receptors to determine the functional significance of some of them. Thus, the use of the snake FSHR may aid the identification of important residues for hormone-receptor interaction and for receptor function regulation.

We also demonstrated that the cloned cDNA was adequately expressed in HEK-293 cells after transient transfections (Figs. 5A and B). First, we showed that the expected 2.5 kb mRNA transcript was present in the cells (Fig. 5A). This 2.5 kb band was shown to encode a complete and functional receptor in in vitro studies with mammalian FSHR (Sprengel et al., 1990). Second, we confirmed the expression of the corresponding protein on the cell surface by using a construct with a FLAG epitope in the aminoterminal domain (Fig. 5B). Furthermore, we demonstrated that the expressed protein was functionally analogous to the mammalian FSHR, since hFSH stimulated the cAMP production in transiently transfected HEK293 cells (Fig. 6). However, the doses of hFSH necessary for stimulation of the recombinant snake FSHR were higher than those needed for stimulation of the rat FSHR. In parallel experiments in HEK 293 cells transiently transfected with the rat FSHR, 100 ng/well hFSH induced a 400% increase in cAMP production (not shown). Since lower doses of mammalian FSH stimulate adenylyl cyclase and cAMP production in lizards (Borrelli et al., 1997, 2000), the lower sensitivity of the snake FSHR expressed in mammalian cells to hFSH may be due to a less efficient transduction apparatus in a heterologous system compared to that activated in homologous systems. However, further studies are necessary to elucidate this topic.

Expression of the endogenous snake FSHR was restricted to gonadal tissues: ovary and testis (Fig. 4). We found the main 2.5 kb transcript, which was also predicted from the nucleotide sequence and a less prominent 3.0 kb transcript in both tissues. A single 2.5 kb transcript was detected in newt testis (Nakayama et al., 2000), while other groups also found multiple FSHR transcripts in gonads of other species such as lizard (Borrelli et al., 2001), chicken (Wakabayashi et al., 1997), and mammals (Gromoll et al., 1993; Yarney et al., 1993). The presence of multiple transcript sizes may be due to alternative transcriptional start sites, differences in polyadenylation sites and alternative splicing processes.

The cloning of gonadotropin receptors of non-mammalian vertebrates is also important for a better understanding of the evolutionary history of this GPCR subfamily. The first non-mammalian gonadotropin receptors were described only recently (Johnson et al., 1996; Mizutani et al., 1998; You et al., 1996) and the only reptilian gonadotropin receptor cloned until now was the lizard FSHR (Borrelli et al., 2001). Complete sequences for LHRs in reptiles or amphibians were not reported so far. We failed to find cDNAs more closely related to LHR in the *B. jararaca* snake, even after repeated attempts using various strategies. It remains to be clarified whether snakes have only one gonadotropin receptor or that their LHR is significantly different from the LHRs currently known.

The phylogenetic analysis clearly showed that the snake gonadotropin receptor groups with FSHRs of other species with a high bootstrap in both MP and NJ (Figs. 3A and B), but it is also clear that the lizard and the snake FSHRs group separately from mammalian FSHRs. Similar results were reported by Borrelli et al. (2001) with respect to the lizard FSHR. Since our main goal was to locate the cloned sequence on the tree topology, here we only presented unrooted trees. For a detailed evolutionary analysis, more internal taxa are needed as outgroups in order to root the trees.

Interestingly, we found a conspicuous difference of branch length involving mammals and squamates in both analysis criteria. Mammals always presented shorter branch lengths than squamates, meaning minor distance in the NJ approach and less steps in the MP analysis. This suggests that evolutionary rate is higher in the Squamata group compared to the mammalian group. Heterogeneous branch lengths were also found inside the other clades and, as expected, the *Drosophila* and the anemone LGRs presented the longest branch lengths.

In conclusion, we cloned, sequenced, and expressed for the first time the snake FSHR. Knowledge about its structure may aid further studies related to structure–function relationships.

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