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ORIGINAL ARTICLE

Molecular cloning and functional analysis of the FSH receptor gene promoter from the volcano mouse (*Neotomodon alstoni alstoni*)

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Abstract To gain further insights on the genetic divergence and the species-specific characteristics of the follicle-stimulating hormone receptor (FSHR), we cloned 946 bp of the 5′-flanking region of the *FSHR* gene from the volcano mouse (*Neotomodon alstoni alstoni*), and compared its features with those from other mammalian species. The sequence of neotomodon FSHR (nFSHR) gene from the translation initiation site to −946 is 74, 71, 64, and 59% homologous to rat, mouse (129/J), human, and sheep, respectively. The nFSHR 5′-flanking region exhibits new interesting putative *cis*-regulatory elements including those for the SRY transcription factor, which had not been previously related to the *FSHR* gene. The transcriptional regulation properties of *nFSHR* gene were studied in mouse Sertoli (MSC-1) and non-Sertoli (H441) cell lines, and compared with those obtained with similar 129/J constructs. All constructs tested were more active in H441 than in MSC-1 cells. The low transcription levels detected in MSC-1 cells probably reflect the recruitment of Sertoli cells-specific nuclear factors that repress transcription of the *FSHR* gene. In H441 cells, 129/J constructs were more active than their neotomodon counterparts, indicating important species-specific differences in their transcription pattern. Functional analysis of a series of progressive 5′-deletion mutants identified regions involved in positive and negative transcriptional regulation as well as the strongest minimal promoter spanning 260 bp upstream the translation initiation site. The identification of inhibitory nuclear transcription factors, which are apparently expressed in MSC-1 cells, may contribute to a better understanding of the transcriptional regulation of the *FSHR* gene.

Keywords Gonadotropin receptors · FSH · *FSHR* gene · Neotomodon · Sertoli cells · Testis

Introduction

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are the pituitary hormones that regulate gonadal function in vertebrates through their interaction with their cognate receptors (R), the FSHR and the LHR. These receptors belong to the superfamily of G protein-coupled receptors (GPCRs), which act through interactions with guanine-nucleotide-binding signal transducing proteins (G-proteins). As other GPCRs, the FSHR contains seven transmembrane domains and a large extracellular domain that interact with the α and β subunits of the FSH. Binding of FSH to its receptor activates the trimeric G_s protein, which stimulates the membrane-bound effector enzyme adenylyl cyclase leading to formation of cAMP and activation of multiple intracellular signaling cascades that influence expression of FSH-dependent genes in the gonads [1].

The cDNA of the FSHR has been cloned from several mammalian and non-mammalian species [2]. These studies have been instrumental not only for a better understanding

The nucleotide sequence data reported in this paper have been submitted to the EMBL nucleotide sequence database under the accession number AM943661.

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of the structure–function relationship of the FSHR [3, 4], but also for the improvement of our knowledge of the phylogenetic relationships and divergence of the FSHR and related receptors. In sharp contrast, the 5′-flanking region of the FSHR has only been cloned from human and sheep, as well as from two rodent species, the rat and mouse [5–8]. These studies have allowed the identification of some transcription factors involved in the transcriptional control of the *FSHR* gene. The nature of the transcription factors so far studied is fundamentally ubiquitous and have been implicated in the basal transcription of the *FSHR* gene [9], while the identification of nuclear factors that control the extraordinary cell specificity and temporal-specific pattern of expression of *FSHR* gene still remains unknown. The study of the FSHR and its gene in other animal species may potentially shed new insights on both the structure–function relationship and the genetic features responsible for its cell-specific pattern of expression.

In the present study, we cloned the 5′-flanking region of the *FSHR* gene from the volcano mouse (*Neotomodon alstoni alstoni*), a particular rodent species endemic to the mountains of the Mexican Transvolcanic Belt, occurring at an altitudinal range of 8,500–14,000 ft. This species has survived for thousands of years due to particular adaptative mechanisms which may have imprinted unique features on its genome [10]. In addition, we compared the structure of the neotomodon 5′-regulatory region and its transcriptional regulatory properties with that of the 129/*JFSHR* gene. The results demonstrate that the 5′-flanking region of the *nFSHR* gene is active in non-Sertoli cells (H441 cell line) and less active in Sertoli cells (MSC-1 cell line), suggesting that the MSC-1 cell line possesses strong inhibitory factors that interact with specific regions of the *nFSHR* gene.

Results

Isolation and sequence analysis of the 5′-flanking region of the *nFSHR* gene

In order to isolate the 5′-flanking region of the *nFSHR* gene, we aligned the 5′-flanking regions of the *FSHR* gene from rat and mouse [6, 7] and found a conserved region localized at −881/−851 bp from the transcription start site of the rat gene. A synthetic oligonucleotide (FSHRp-fw3) containing this sequence was used as primer for the amplification of neotomodon genomic DNA by PCR. The downstream primer was an oligonucleotide (FSHRp-rev1) corresponding to the sequence +122/+93 of the hamster FSHR cDNA [11]. This latter species was chosen considering that the neotomodon and hamster belong to the same taxonomic family (*Cricetidae*). An amplified product of approximately 1.1 kb was isolated, cloned, and sequenced (Fig. 1). To confirm the fidelity of the sequence, two other independent clones were completely sequenced. The nFSHR 5′-flanking region contains a sequence (Fig. 1, −163/−139) that resembles the A/T-rich region found in many TATA-containing promoters. Nevertheless, it does not perfectly match the well defined consensus sequence (TATAAA) of the canonical TATA box. The DNA cloned also includes the nucleotide sequence that encodes the first 31 amino acids of the nFSHR; it spans the complete signal peptide (17 residues) and the first 14 amino acids of the mature protein.

We also cloned and analyzed the 5′-flanking region of the 129/J FSHR (129/JFSHR) gene following the above mentioned strategy. Although the nucleotide sequence of the 129/*JFSHR* gene is almost identical to that identified in the Balb/c mouse by Huhtaniemi et al. [7] (Accession No. S49632), we detected a nucleotide change (A/G) at position

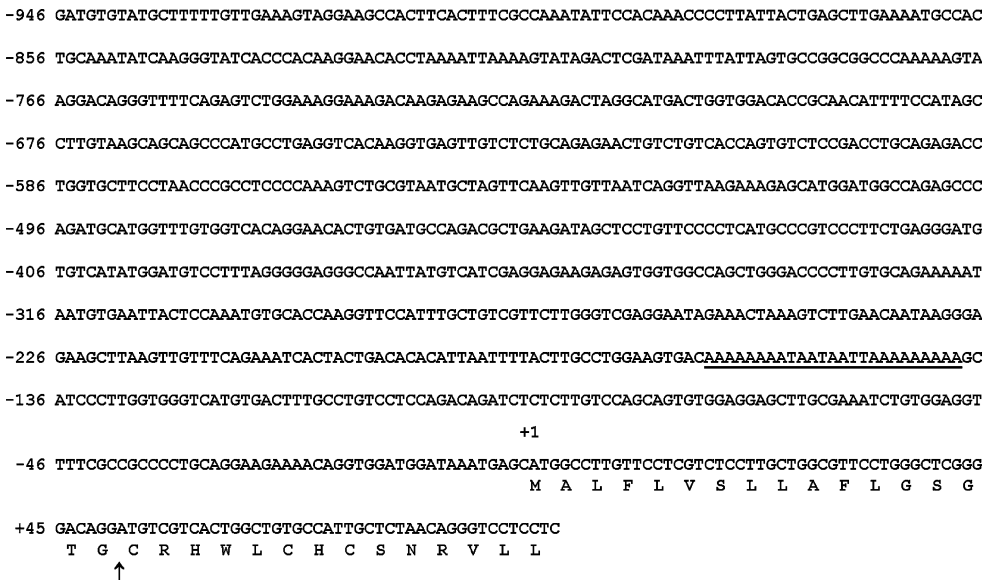

Fig. 1 Nucleotide sequence of the 5′-flanking region of the neotomodon *FSHR* gene. Nucleotides are numbered from the translation initiation site, which is indicated by +1. The predicted amino acid sequence partially encoded by exon 1 is indicated below the nucleotide sequence. The A/T rich region is *underlined* and the putative cleavage site of the signal peptide is indicated by a *vertical arrow*

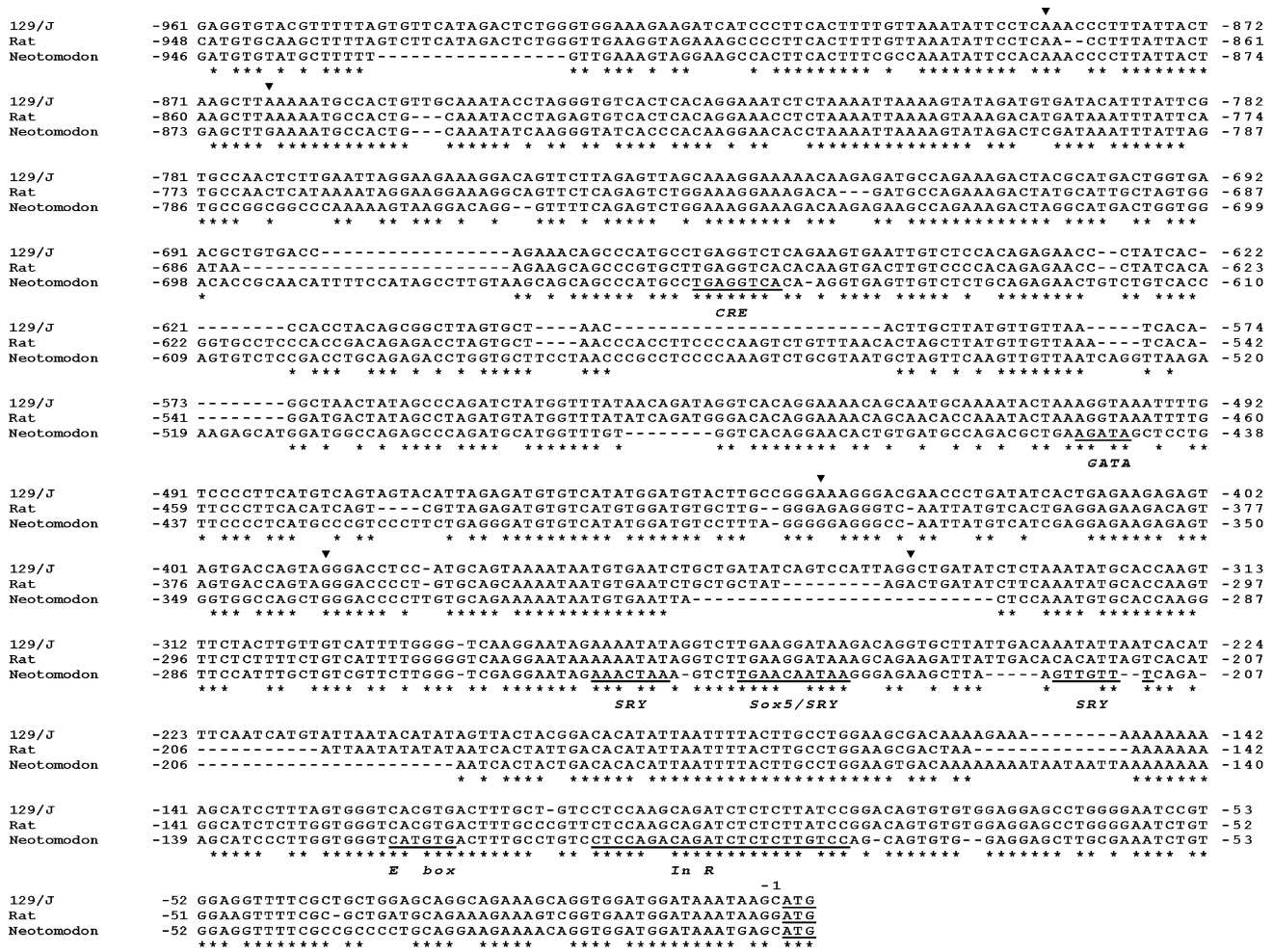

Fig. 2 Alignment of the promoter regions of neotomodon, 129/J, and rat *FSHR* gene. The nucleotide differences between the 129/J FSHR promoter and that reported by Huhtaniemi et al. [7] are indicated with *arrow heads*. Putative relevant transcription factor binding sites are *underlined* and *labeled*. Conserved nucleotides (*) and the translation initiation site ATG (*underlined*) are indicated

−339 and four single nucleotide insertions at positions −390, −436, −865, and −886 (Fig. 2). A comparison between the sequence shown in Fig. 2 and that covering the C57BL/6J mouse chromosome 17 (Accession No. AC165082) revealed that only the (A/G) substitution at position −339 remained present. The differences detected may be due to the particular mouse strain employed to obtain the FSHR 5′-flanking region.

Comparative analysis of the nucleotide sequences of the FSHR promoter

Alignment of the neotomodon FSHR 5′-flanking region with two other phylogenetically related rodent species [129/J and rat *FSHR* gene [6] (Accession No. S81117)] indicated their evolutionary relationship (Fig. 2). The sequence of the *nFSHR* gene from the translation initiation site to −946 is 74 and 71% homologous to rat and 129/J, respectively; this homology decreases to 64 and 59% for less related species such as human [5] and sheep [8], respectively (not shown).

We also detected high sequence homology with the E box and the initiator region (In R) previously described for the rat *FSHR* gene [12]. However, a 1-bp change in the neotomodon E box (Fig. 2) was noticed. This change disrupts the palindromic structure of the E box and probably affects negatively the binding of USF transcription factors since bases within the core of the E box are critical for recognition and function of the rat element [13]. The sequence around the E box (GGTCAtgTGAC) (Fig. 2, −126/−116) has also been identified as a putative estrogen responsive element (ERE) in the human and ovine FSHR promoters [5, 8]. This *cis*-element, however, failed to act as an ERE in the neotomodon promoter since no change in promoter activity was detected when estrogen receptor-positive MCF-7 cells transiently transfected with the reporter plasmid pnFSHR-946 were stimulated with either vehicle or 17β-estradiol (1×10^{-8} M) (data not shown).

Neither the GATA 1 binding site (TATC) in the rat gene [14] nor the COUP-TFs binding site in the ovine gene [15] are conserved in the neotomodon promoter.

We then examined the nFSHR promoter for putative transcription factor binding sites. Although binding sites were predicted for a number of transcription factors, only few of them are conserved in the three rodent species analyzed (Fig. 2). The CRE-like sequence, which may be involved in the regulation of *FSHR* gene expression by cAMP [16, 17], is well conserved in the three rodent species examined. Noteworthy is a cluster of three SRY binding sites in the nFSHR promoter, which is absent in the mouse and rat counterparts. The presence of SRY binding sites within the nFSHR promoter is of potential interest given the fundamental role of SRY expression in Sertoli cell differentiation [18].

Comparative analysis of the FSHR promoter activity

The activity of the nFSHR promoter in mouse Sertoli (MSC-1) and non-Sertoli (H441) cell lines was analyzed. The H441 cell line was isolated from a human lung adenocarcinoma and has been previously used to identify *cis*-acting elements and *trans*-acting factors implicated in the regulation of lung-specific genes [19, 20]. Luciferase reporters driven by various regions of the nFSHR promoter were transiently transfected into the above mentioned cell lines and their activities were compared to those of similar 129/J constructs (based upon homology). The results shown in Fig. 3 indicate that in MSC-1 cells the relative luciferase activity of p129/JFSHR-961 reporter was even lower than that obtained with the vector alone. Removal of the first 96 bp (p129/JFSHR-865) resulted in no change in luciferase activity. Meanwhile, removal to −552 bp increased the activity of the luciferase reporter to 2.7-fold the activity of p129/JFSHR-865 ($P < 0.05$), suggesting that cell-specific inhibitory factors bind to the deleted region. Similar results were obtained with neotomodon constructs; however, the inhibitory region seemed to be located between −946 and −868 bp (see below). Unexpectedly, all luciferase-reporters were active in the H441 cell line, indicating that they are transcriptionally competent. In this cell line the p129/JFSHR-961 and −552 constructs were ∼60% more active than their neotomodon counterparts, whereas the p129/JFSHR-865 and pnFSHR-867 constructs showed similar reporter activities.

Functional analysis of the nFSHR promoter

To further characterize the critical promoter sequence in the 5′-flanking region of the nFSHR, a series of deletion mutants were also transiently transfected into MSC-1 and H441 cells. In H441 cells, the 5′-flanking DNA fragment between −946 and −1 bp exhibited a 4.3-fold increase over the promoterless control plasmid, whereas in Sertoli cells (MSC-1) the activity was lower than that exhibited by the vector alone (Fig. 4). Deletion of the region located between −946 and −867 bp led to a decrease (25.4%, $P < 0.05$) of promoter activity in H441 cells but to a significant ($P < 0.05$) increase (8.4-fold the activity of pnFSHR-946) in MSC-1 cells, suggesting that interaction of Sertoli cell-specific proteins with *cis*-element(s) within

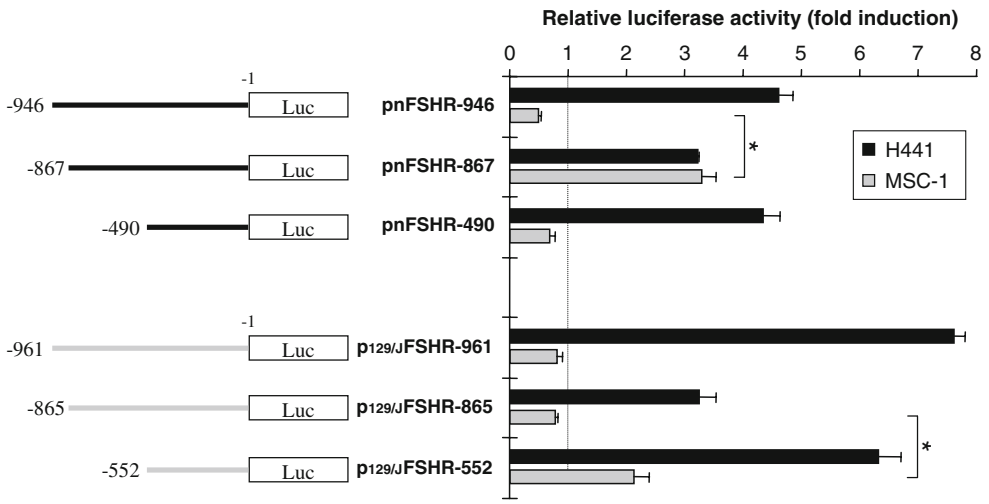

Fig. 3 Transcriptional activity of the 5′-flanking regions of the nFSHR and 129/*JFSHR* gene. Schematic of the luciferase reporter constructs are shown on the *left*. Fragments cloned into a luciferase reporter vector were cotransfected with the pRL-TK plasmid (*Renilla* luciferase) into mouse Sertoli (MSC-1) and non-Sertoli (H441) cell lines and their relative activity (luciferase/*Renilla* luciferase) was determined in cell lysates 48 h later. Relative luciferase activity is expressed as fold induction assuming that the activity of the promoterless plasmid pGL3B is 1. Data are means ± SD ($n = 3$) from one representative experiment. Differences in transcriptional activity were calculated by the Student's *t*-test. * $P < 0.05$

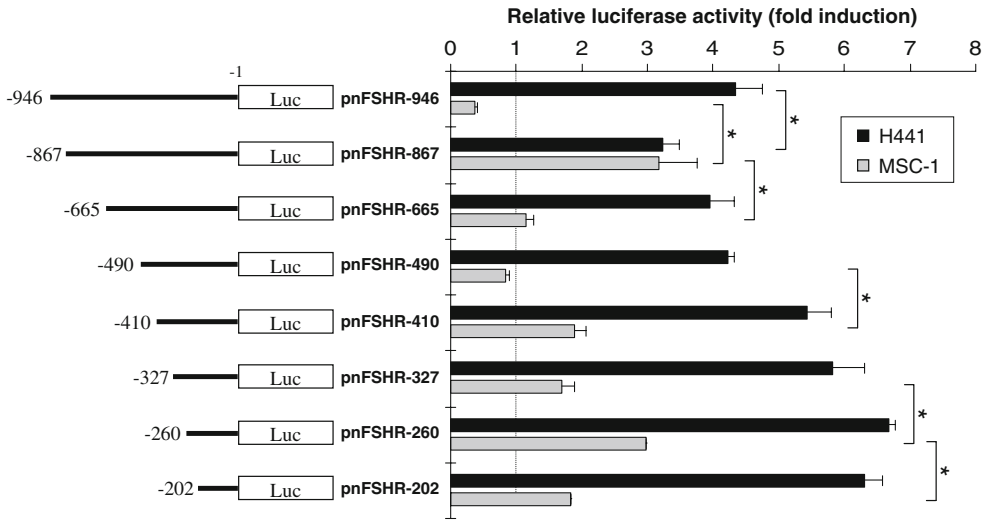

Fig. 4 Activity of the 5′ deletions mutants of the *nFSHR* gene in different cell lines. The left side of the figure schematically shows the luciferase reporter constructs. Each construct was cotransfected with the pRL-TK plasmid (*Renilla* luciferase) into mouse Sertoli (MSC-1) and non-Sertoli (H441) cell lines and their relative activity (luciferase/*Renilla* luciferase) was determined in cell lysates 48 h later. The relative luciferase activity is expressed as fold induction assuming that the activity of the promoterless plasmid pGL3B is 1. Values represent means ± SD ($n = 3$) from one representative experiment. Differences in transcriptional activity were calculated by the Student's *t*-test. * $P < 0.05$

this region probably repressed transcription of the *nFSHR* gene. Deletion to −665 bp reduced (63.5%, $P < 0.05$) luciferase activity in MSC-1 cells indicating that important positive transcription factor(s) interact with the −867/−665 promoter region, while in H441 this deletion restored promoter activity. Deletion to −490 bp further decreased promoter activity in MSC-1 cells, whereas in H441 cells no obvious change was detected. Further deletions of the promoter sequence to −410, −327, −260, and −202 bp increased promoter activity in H441 cells to a maximum of 6.6-fold over the promoterless plasmid (Fig. 4, pnFSHR-260), whereas in MSC-1 cells the same genetic constructions identified two additional, less strong inhibitory regions at −490/−410 and −327/−260 bp, as well as a region at −260/−202 bp involved in positive regulation. In general, shorter DNA constructs were more active than the longer constructs in both cell lines, which is in agreement with observations in FSHR promoters from other species [5, 13, 21].

Discussion

Despite the major regulatory role of the FSHR on reproductive function in vertebrate species, the mechanisms underlying the cell-specific expression and regulation of this particular receptor in the gonads are poorly understood. In the present study, we isolated the 5′-flanking region of the *FSHR* gene from the volcano mouse (*Neotomodon alstoni alstoni*) and compared the identified sequence with those previously described for other species. The nFSHR 5′-flanking region shares considerable sequence homology with those from other rodents (Fig. 2), followed by human and sheep. The neotomodon promoter contains some conserved regulatory elements, including that previously described as a putative estrogen responsive element (ERE) in the human and ovine *FSHR* gene [5, 8]. However, this putative *cis*-element failed to act as an ERE in the neotomodon promoter, which may be probably due to the lack of the third nucleotide between the palindromic sequence. Other conserved regulatory elements also include an E box, which binds upstream stimulatory factor 1 (USF1) and USF2 in vitro [12, 13, 21] and in vivo [22, 23] as well as a CRE-like sequence probably involved in cAMP mediated transcriptional regulation [16]. The neotomodon FSHR promoter also contains novel putative *cis*-acting elements for transcription factors of the HMG-box SRY/Sox family, which seem to be important in the positive transcriptional regulation of the *nFSHR* gene, since elimination of the region containing these putative SRY binding sites significantly reduced reporter activity. The high score to the SRY consensus matrix and the importance of the SRY transcription factor in determining Sertoli cell phenotype [18] additionally support its possible role on the transcriptional regulation of the nFSHR promoter.

Transient transfection experiments with several genetic constructs indicated that the nFSHR promoter was more effective in non-Sertoli (H441) than in Sertoli (MSC-1) cells. The transcriptional activity detected in H441 cells indicates that the promoter function of the 5′-flanking

region of the nFSHR is apparently less efficient in the MSC-1 Sertoli cell line, as disclosed by the low transcription levels detected in this latter cell type. In some instances, however, the promoter activity in MSC-1 cells was as active as in the non-Sertoli cell line H441 (Fig. 3, see pnFSHR-867) indicating that cell-specific inhibitory nuclear factor(s) rather than ubiquitous transcription factor(s) attenuated the FSHR promoter activity. This observation is in line with the low transcription levels observed in the Sertoli cell line 15P1 transiently transfected with the ovine *FSHR* gene promoter [21].

Comparison of nFSHR promoter activity with that from the 129/J unveiled remarkable similarities and some interesting differences in their transcription patterns. Although the 129/J FSHR promoter also showed preferential expression in non-Sertoli cells (H441), the transcriptional activity of two 129/J constructs (p129/JFSHR-961 and p129/JFSHR-552) were higher ($\sim$60%) than that of their corresponding neotomodon counterparts. These data indicate the existence of subtle differences in the mouse *cis*-acting elements that determine their higher activity, thus underlining the importance of species differences in the function of this particular promoter.

Transient transfection experiments with a series of deletion mutants allowed to identify more precisely several transcriptional regulatory regions in the nFSHR promoter. In MSC-1 cells, three regions involved in negative transcriptional regulation were identified at −946/−867, −490/−410, and −327/−260 bp; these regions may play an important role in determining low transcription levels in this particular cell line. Noteworthy is the region between −946 and −867 bp, which strongly impaired promoter activity. In fact, strong inhibitory regions in the *FSHR* gene promoter have also been detected in several gonadal cell lines [5, 13, 21, 24].

Collectively, these results strongly suggest that the MSC-1 cell line possesses cell-specific transcription factors that negatively regulate the transcription of the FSHR promoter. Determining these factors will contribute to unveil the positive transcriptional regulation of this particular receptor promoter, which until now has remained elusive.

Materials and methods

Animals

Young adult strain 129/J mice (*Mus musculus*) and volcano mice (*Neotomodon alstoni alstoni*) aged 5 months were obtained from the animal care facility of the Universidad Nacional Autónoma de México (México D.F., Mexico). Livers, obtained immediately after killing, were cut into small pieces and stored at −70°C until DNA preparation.

Genomic DNA cloning

DNA was isolated from the liver of 129/J and neotomodon as described previously [25], and quantified by spectrophotometry at 260 nm. Cloning of the FSHR 5′-flanking regions was carried out by PCR using genomic DNA from each species as template. DNA was amplified using the forward (FSHRp-fw3) and reverse (FSHRp-rev1) oligonucleotides as primers, shown in Table 1. Reactions were subjected to 30 cycles (30 s at 94°C, 2 min at 52°C, and 3 min at 72°C), followed by a final extension cycle at 72°C for 10 min. The PCR reactions were analyzed by agarose gel electrophoresis and the DNA bands of $\sim$1100 bp were excised, recovered (Gene Clean Kit, Bio 101, Carlsbad, CA, USA), and cloned into the pGEM-T vector (Promega, Madison, WI, USA) to obtain the recombinant plasmids designated as pnFSHR1.1 (neotomodon) and p129/JFSHR1.1 (mouse). The genomic DNA inserts from three different clones of each species were sequenced on both strands.

Sequence analysis

The identity of the nucleotide sequence of the 5′-flanking region of the *nFSHR* gene was confirmed with the BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi). The FSHR promoter regions from different species were aligned with ClustalW2 program [26] (www.ebi.ac.uk/Tools/clustalw2/index.html), while the putative transcription binding sites were predicted with the TFSEARCH program [27] (www.cbrc.jp/research/db/TFSEARCH.html).

Reporter plasmids

The 5′-flanking region of the *nFSHR* gene spanning specific nucleotides was amplified by PCR using the pnFSHR1.1 plasmid as template. The neotomodon (n) specific primers used for PCR amplification are shown in Table 1. The same procedure was used to obtain the 129/J FSHR reporter plasmids [using the p129/JFSHR1.1 plasmid and the 129/J specific oligonucleotides shown in Table 1]. The PCR products were ligated into the pGEMT vector and transformed in the *E. coli* JM109 strain (Promega). The recombinant plasmids were digested with *Xho*I and *Nco*I, and the released fragment was inserted into the luciferase reporter vector pGL3-Basic (pGL3B) (Promega). Reporter plasmids were transformed in JM109 cells and purified by anion-exchange chromatography (Qiagen, Valencia, CA, USA). Plasmid DNAs were quantified by measuring the absorbance at 260 nm and their quality was verified by agarose gel electrophoresis. The orientation and fidelity of all constructs were confirmed by automated DNA sequencing.

Table 1 Sequence of the oligonucleotides used in this study

Name	Sequence	Size (bp)	Relative position	Application	Restriction site added
FSHRp-fw3	5′-GCAGGTAATAAGTTGTAGACATGATCTTAGA-3′	31	(−881/−851)[a]	Genomic DNA cloning	
FSHRp-rev1	5′-GGAATCTCCGTCACCTTGCTGTCTTGGCAG-3′	30	(+122/+93)[b]	Genomic DNA cloning	
nFSHR-946	5′-CTCGAGGATGTGTATGCTTTTTGTTG-3′	26	(−946/−927)[c]	Reporter construct	*Xho*I
nFSHR-867	5′-CTCGAGGAAAATGCCACTGCAAATATC-3′	27	(−867/−847)[c]	Reporter construct	*Xho*I
nFSHR-665	5′-CTCGAGCAGCCCATGCCTGAGGTCAC-3′	26	(−665/−646)[c]	Reporter construct	*Xho*I
nFSHR-490	5′-CTCGAGATGGTTTGTGGTCACAGG-3′	24	(−490/−473)[c]	Reporter construct	*Xho*I
nFSHR-410	5′-CTCGAGGATGTGTCATATGGATGTCC-3′	26	(−410/−391)[c]	Reporter construct	*Xho*I
nFSHR-327	5′-CTCGAGTGCAGAAAAATAATGTGAATTAC-3′	29	(−327/−305)[c]	Reporter construct	*Xho*I
nFSHR-260	5′-CTCGAGAGGAATAGAAACTAAAGTCTTG-3′	28	(−260/−239)[c]	Reporter construct	*Xho*I
nFSHR-202	5′-CTCGAGACTACTGACACACATTAATTTTAC-3′	30	(−202/−179)[c]	Reporter construct	*Xho*I
nFSHR-rev	5′-CCATGG tgGCTCATTTATCCATCCAC-3′*	26	(−1/−18)[c]	Reporter construct	*Nco*I
129/JFSHR-961	5′-CTCGAGGAGGTGTACGTTTTTAGTG-3′	25	(−961/−943)[d]	Reporter construct	*Xho*I
129/JFSHR-865	5′-CTCGAGAAAAATGCCACTGTTGCAAATAC-3′	29	(−865/−843)[d]	Reporter construct	*Xho*I
129/JFSHR-552	5′-CTCGAGATGGTTTATAACAGATAG-3′	24	(−552/−535)[d]	Reporter construct	*Xho*I
129/JFSHR-rev	5′-CCATGGtgGCTTATTTATCCATCCAC-3′*	26	(−1/−18)[d]	Reporter construct	*Nco*I

[a] Rat FSHR 5′-flanking region (Heckert et al. [6])

[b] Hamster FSHR cDNA (Zhang and Roy [11])

[c] Neotomodon FSHR 5′-flanking region (Fig. 1)

[d] 129/J FSHR 5′-flanking region (Fig. 2)

* Nucleotides in lowercase letters were added to conform a Kozak consensus sequence. Restriction sites are *underlined*

Cell culture, transfections, and luciferase assays

NCI-H441 [28] and MSC-1 [29] cells were cultured in RPMI 1640 and DMEM (Gibco-Invitrogen, Grand Island, NY, USA), respectively, supplemented with 10% fetal bovine serum (Gibco). The cell lines were cultured in the absence of antibiotics and maintained in a humidified incubator at 37°C under 5% CO_2. Thirty thousand cells per well of each cell line were plated in 48-well plates 18–24 h before transfection. Plasmid DNAs were transiently transfected using Effectene (Qiagen), following the instructions recommended by the manufacturer. Cells were cotransfected with the *Renilla* luciferase expression plasmid pRL-TK (Promega) to normalize for transfection efficiency. For each well, 150 ng of the reporter construct and 50 ng of pRL-TK were used. After a 48 h incubation period, cells were lysed and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). pGL3B was used as background control. Three independent transfections were performed in triplicate.

Statistical analysis

Differences in transcriptional activity were calculated by one-way analysis of variance (ANOVA) followed by the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

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