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Full Length Research Paper

Molecular cloning and functional analysis of the follicle-stimulating hormone (FSH) receptor gene promoter from the Jintang black goat

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A 762 bp fragment of the 5'-flanking region of the FSHR gene from the Jintang black goat was cloned. The putative initial transcript site was the A at 681 bp and there were 7 putative *cis*-acting elements and 3 AT-rich regions. The sequence of the *FSHR* promoter from the Jintang black goat is 99.34% homology to *Capra hircus*, 32.38% to *Gallus gallus* and 38.55% to mouse. It could promote the EGFP, FSHR transcription in HEK293 cells, the fluorescence intensity was weaker than the CMV promoter, but the expressed FSHR could respond to the FSH signaling with signal intensity much higher than that at 24 h. This indicated that the *FSHR* promoter of the Jintang black goat is a strong promoter and may be a gene-special promoter.

Key words: Follicle-stimulating hormone receptor, gene promoter, Jintang black goat, molecular cloning, functional analysis.

INTRODUCTION

The Jingtang black goat is an excellent local goat breeds in Chengdu, China. It grows fast and its body size is large. Specially, its reproduction rate (242%) is higher than other goat breeds (Boer goat 198%, Nanjiang yellow goat 219%, etc.) (LiJian, 2005). Researchers have been looking for the key genes that influence the high reproductive rate and have made some progresses in sheep. Three major genes (*FecB* (Souza et al., 2001), *BMP15* (Galloway et al., 2000) and *GDF9* (Juengel et al., 2002)) were identified in different breeds of sheep to inhibit the expression of the FSH receptor or the interaction of FSHR with its downstream effectors (Shimasaki et al., 2004). So, the expression of FSHR is the most important factor in the reproduction traits determinants.

In our previous study, we found that 2 FSHRs (FSHRA and FSHRB) were expressed in the Jintang black goat simultaneously (data to be published). They might be alternatively spliced by the same primary mRNAs, enhance the FSH signal through the hetero-dimerization and lead to high FSH signal response. The expression amounts of FSHRA and FSHRB in the reproduction organs are not equal, as this, further research of the FSHR gene expression is needed.

FSHR belongs to the family of G protein-coupled receptors, complex transmembrane proteins characterized by seven hydrophobic helices inserted in the plasmalemma. The intracellular portion of the FSHR is coupled to a Gs protein and upon receptor activation by the hormonal interaction with the extracellular domain, the cascade of events that ultimately leads to the specific biological effects of the gonadotropin would be initiated (Simoni et al., 1997; George et al., 2011).

Promoter is the most important regulatory element in the gene expression. Since Gromoll et al. (1994) cloned the human FSHR 5'-UTR in 1994, many FSHR promoters

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Abbreviation: FSH, Follicle-stimulating hormone; **FSHR,** follicle-stimulating hormone receptor.

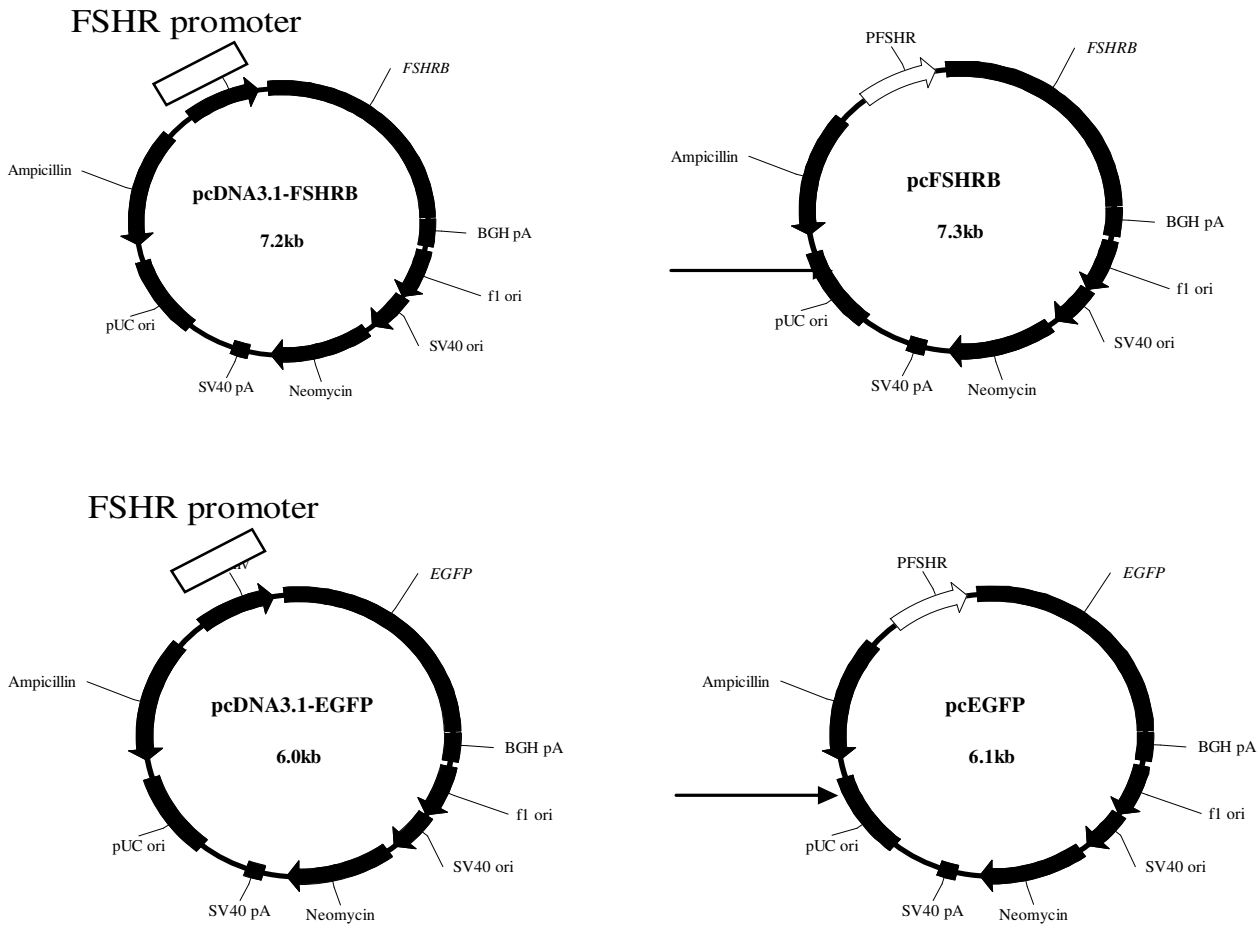

Figure 1. Construction of expression plasmids pcFSHRB and pcEGFP. Based on the expression plasmids pcDNA3.1-FSHRB and pc DNA3.1-EGFP, their CMV promoters were replaced by the FSHR promoter of the Jintang black goat.

have been cloned from different species (Levallet et al., 2001; Zaidi et al., 2007; Perez-Solis et al., 2010). But till now, the regulatory pattern of the FSHR expression still remains unknown. To understand the pattern more, we cloned and analyzed the FSHR promoter of the Jintang black goat.

MATERIALS AND METHODS

Animals

Adult Jintang black goat aged 3 years was obtained from the Jintang black goat breeds farm, Chengdu, China.

Plasmids, strain, cells and primers

pcDNA3.1-EGFP, pcDNA3.1-FSHRB were constructed by inserting *EGFP*, *FSHRB* in the MCS of pcDNA3.1(+), in phase shown in Figure 1. *Escherichia coli* JM109 (*F´traD36 proA+B+ laclq Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17)* was stored in our laboratory. HEK293 cells were obtained from ATCC (American type culture collection). All primer used in this study are listed in Table 1.

FSHR promoter cloning

DNA was isolated from the ovary of the Jintang black goat as described previously (Sambrook, 2001) and quantified by spectro-photometry at 260 nm. Cloning of the FSHR promoter was carried out by PCR using genomic DNA as template. Reactions were subjected to 30 cycles (30 s at 94°C, 2 min at 55°C and 1 min at 72°C, followed by a final extension cycle at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and the DNA band of 762 bp was recovered and cloned into the pMD-18T vector (TAKARA, Dalian, China).

Sequence analysis

The FSHR promoter regions from different species were aligned with ClustalW2 program (Larkin et al., 2007) (www.ebi.ac.uk/Tools/clustalw2/index.html), while the putative transcription binding sites were predicted with the TFSEARCH program (Heinemeyer et al., 1998) (www.cbrc.jp/research/db/TFSEARCH.html). Initial transcript site was predicted by BDGP (Reese 2001) (http://www.fruitfly.org/seq_tools/promoter.html).

Table 1. Shows the primer sequences used in this study.

Name	Type	Sequence (5'-3')	Note
F1	Sense	TTCCGACAAGGCAAAACGG	Used for promoter cloning.
R1	Antisense	CATGTTTACTTATCCATCCACCTGC	
F2	Sense	CGATGTACGGGCCAGATATACGCTTC CGACAAGGCAAAACGGAC	Used for plasmid construction, amplification the promoter sequence, underlined sequence defines the sequence of backbone.
R2	Antisense	TAAGCAGTGGGTTCTCTAGTTAGCCA GACCACACTGAGCCCTTTTGAG	
F3	Sense	CTCAAAAGGGCTCAGTGTGGTCTGG CTAACTAGAGAACCCACTGCTTA	Used for plasmid construction, amplification the backbone, underlined sequence defines the sequence of backbone.
R3	Antisense	GTCCGTTTTGCCTTGTCGGAAGCGT ATATCTGGCCCGTACATCG	

Expression plasmids construction

The expression plasmids pcFSHRB, pcEGFP were constructed as described previously (Li and Evans, 1997). The expression plasmids pcDNA3.1-FSHRB, pcDNA3.1-EGFP were used as template to amplify the backbone of pcFSHRB, pcEGFP. The pMD-18T-FSHR was used as template to amplify the promoter. PCR reaction was carried out to 30 cycles (30 s at 94°C, 30 s at 55°C and 6 min at 72°C, followed by a final extension cycle at 72°C for 10 min). The promoter, backbone fragments were mixed in equal molar ratio, digested using T4 DNA polymerase, annealed at 75°C, then transformed into *E. coli.* JM109. The positive clones were screened and identified by PCR. The expression plasmids were extracted from the positive clones as usual (Sambrook, 2001).

Cell culture and transfection

HEK 293 cells were maintained in DMEM (high glucose) medium containing 10% fetal bovine serum. HEK293 cells in each well of 12 wells plate were transfected (TRANSfection,Tiangen, China) with 1 μg plasmids in DMEM (high glucose) for the indicated times.

cAMP assay

cAMP concentrations were measured using a radioimmunoassay kit (China Atomic Energy Research Institute, Beijing, China), according to the manufacturer's instructions.

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.

RESULTS

Isolation and sequence analysis of the 5'-flanking region of the FSHR gene

Based on the sequence of *Capra hircus* follicle-stimulating hormone receptor promoter region (AY765375.1), a pair of primers were designed and used to amplify the 762 bp fragment of FSHR promoter from the Jintang black goat genomic DNA (Figure 2).

Sequence analysis displays that the putative initial transcript site is the A of GGA located at 681 bp of this fragment and there are 7 putative *cis*-acting elements, TATA-box at -91 bp, CCAAT-box at -205 bp, GC box at -154 bp, USF at -49 bp, E-box at -45 bp, 2 GATA-boxes at -339 bp, -176 bp in this fragment respectively and there are 3 AT-rich region located at -381, -256 and -83 bp in this fragment. These *cis*-elements are non-typical and TATA-box, CCAAT-box and GC box are not located at -25 to -30 bp, -75 to -80 bp and -100 to -150 bp.

Comparative analysis of the nucleotide sequences of the FSHR promoters

Alignment of FSHR promoter sequence of the Jintang black goat with that of *C. hircus*, showed that the homology is 99.34% and there are only 5 bp changes (Figure 3). And these changes do not occur at the *cis*-acting elements, so it is suggested that this does not influence the promoter activity.

Alignment of FSHR promoter sequence of the Jintang black goat with that of *Gallus gallus* and mouse displayed that the homology between them is 32.38 and 38.55%, respectively. This indicates that the difference of FSHR promoter sequence in the same species is much lower than that between different species.

Exogenous gene transcription activity analysis of the Jintang black goat FSHR promoter

To clarify the FSHR promoter activity of the Jintang black goat, we replaced the CMV promoter of the pcDNA3.1-EGFP by *FSHR* promoter, which resulted in the expression of plasmid pcEGFP. The stated 2 plasmids were used to transfect HEK293 cells, respectively and the

Figure 2. Nucleotide sequence of the 5'-flanking region of the Jintang black goat's FSHR gene. Nucleotides are numbered from the initial transcription site, which is indicated by +1. The AT rich region is wave underlined, the E-box is dot underlined and the putative *cis*-acting elements are in box. The translation initiation site is indicated by vertical arrow.

```
Jintang_black_go TTCCGACAAGGCAAAACGGACACGTGACCGAGGGTGGGATAGTTGGTGTCTACTAAGCCC    60
Capra_hircus     ------------------------------------------------------------    60

Jintang_black_go AAAAACCCAGCCTGACCTGTTGGTCACTCAGCTGAGTCAGTTATCTCTGTAGACTACTCT   120
Capra_hircus     ------------------------------------------------------------   120

Jintang_black_go TAAAAGTGCCTCCTACCATCTGTCCAGGGGCTCACTAACCCACTGCCTGTCTTCTGAGCT   180
Capra_hircus     ---------------------------------------------------------..--   178

Jintang_black_go ACACCATATTTGGTTGTTAATTCCAGCAAGAAAGAGAATTAGTGGCTTTGACCCAGAAGT   240
Capra_hircus     ------------------------------------------------------------   238

Jintang_black_go TCTGGTTTTGTATCAAGC.GCCTGGAGGAAGACATTGACACCAAGACTGGAAACAGGTCC   299
Capra_hircus     ----------------a-------------------------------------------   298

Jintang_black_go CTGACCTTCACTGAGGAACTCTGTTAATGATGTTTCACACTGCAGATTGCATCTGTTTTG   359
Capra_hircus     ------------------------------------------------------------   358

Jintang_black_go GAGAAAGTCAAGCGTGTCACTCTGTTTTGAGAAGAAAAAAAAAAAAGTGACCCACAGGGAC   419
Capra_hircus     --------------------------------a---------------------------   418

Jintang_black_go AGTCTTACAGCGAATTTAATATAAGCTATTCTAGACATGCATCAAGTTTCAATTTGCAAA   479
Capra_hircus     ------------------------------------------------------------   478

Jintang_black_go CCCAACCAAAAAGGGTAAAGGGACAGCGTATCTTGCC.CGCCCTCTACCTCTCCCTCCCC   538
Capra_hircus     ------------------------------------a-------------------------   538

Jintang_black_go ACCCCCACCAAAGTCACTGCTGTCACTCAGAAATTCTGCTATTTGTCTGGAAGTGACCGA   598
Capra_hircus     ------------------------------------------------------------   598

Jintang_black_go TAAAAAAGAAAAAAAGGAACACGGCCCTGGGCGGGTCACGTGACCCTACCAGCTCCCAAC   658
Capra_hircus     ------------------------------------------------------------   658

Jintang_black_go GCAGACCTCTTCTCAAAAGGGCTCAGTGTGGAGCCTCTGAAATCTGGGCAGGATTGTCTC   718
Capra_hircus     ------------------------------------------------------------   718

Jintang_black_go TGCAGAGGCAGAAGCAAGCAGGTGGATGGATAAGTAAACATG                    760
Capra_hircus     -----------------------------------------                    760
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Figure 3. Alignment of the promoter regions of the Jintang black goat, *C. hircus* (GenBank: AY765375.1). The dot is the lacked nucleotide.

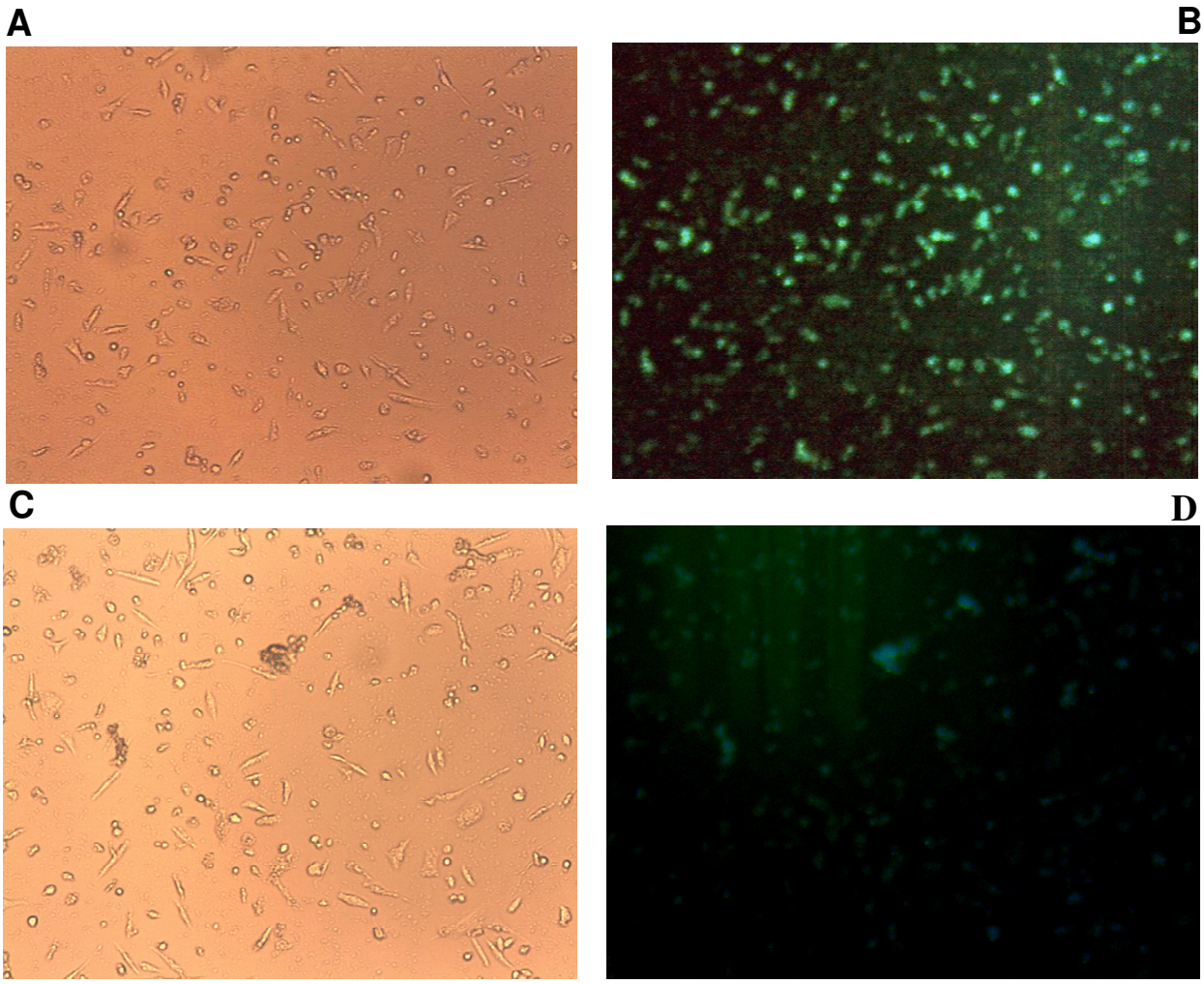

Figure 4. Green fluorescence in HEK293 cells observed under fluorescence microscope. (A, B) pcDNA3.1-EGFP transfected HEK293 cells; (C, D) pcEGFP transfected HEK293 cells; (A, C) in light fields; (B, D) in dark fields.

transfected cells were observed under the fluorescent microscope for green fluorescense after 24 h growth (Figure 4). It was found that the fluorescence was detected in the pcEGFP-transfected cells. This indicates that the FSHR promoter of the Jintang black goat can promote the exogenous gene transcription and has the promoter activity.

But compared with the pcDNA3.1-EGFP-transfected cells, the fluorescence intensity of the pcEGFP transfected cells was weaker. This indicates that the CMV promoter activity is stronger than the FSHR promoter of Jintang black goat for the exogenous gene transcription in HEK293 cells.

FSHR transcription activity analysis of the Jintang black goat FSHR promoter

We replaced the CMV promoter of the pcDNA3.1-FSHRB by FSHR promoter and resulted in the expression of plasmid pcFSHRB. These 2 plasmids were used to transfect HEK293 cells, respectively. After 24 h, 2 mIU/ml FSH was added into the medium and then the cells were cultured for additional 24 and 48 h. The cells were collected and cAMP concentration was determined. The HEK293 cells were set as control.

The cAMP concentration of pcFSHRB-transfected cells is 299.5813±12.4906 and 120.0571±11.0999 pmol/l at 24 and 48 h. However, the cAMP concentration of pcDNA3.1-FSHRB-transfected cells is 125.5281±8.0838 and 109.9407±10.9578 pmol/l at 24 and 48 h (Figure 5). The cAMP production of the pcFSHRB-transfected cells is significantly higher than that of pcDNA3.1-FSHRB at 24 h. This indicates that the transcription activity of the FSHR promoter of the Jintang black goat is higher than that of CMV promoter for the FSHR. And the cAMP concentration reached the peak value at 24 h and then fell

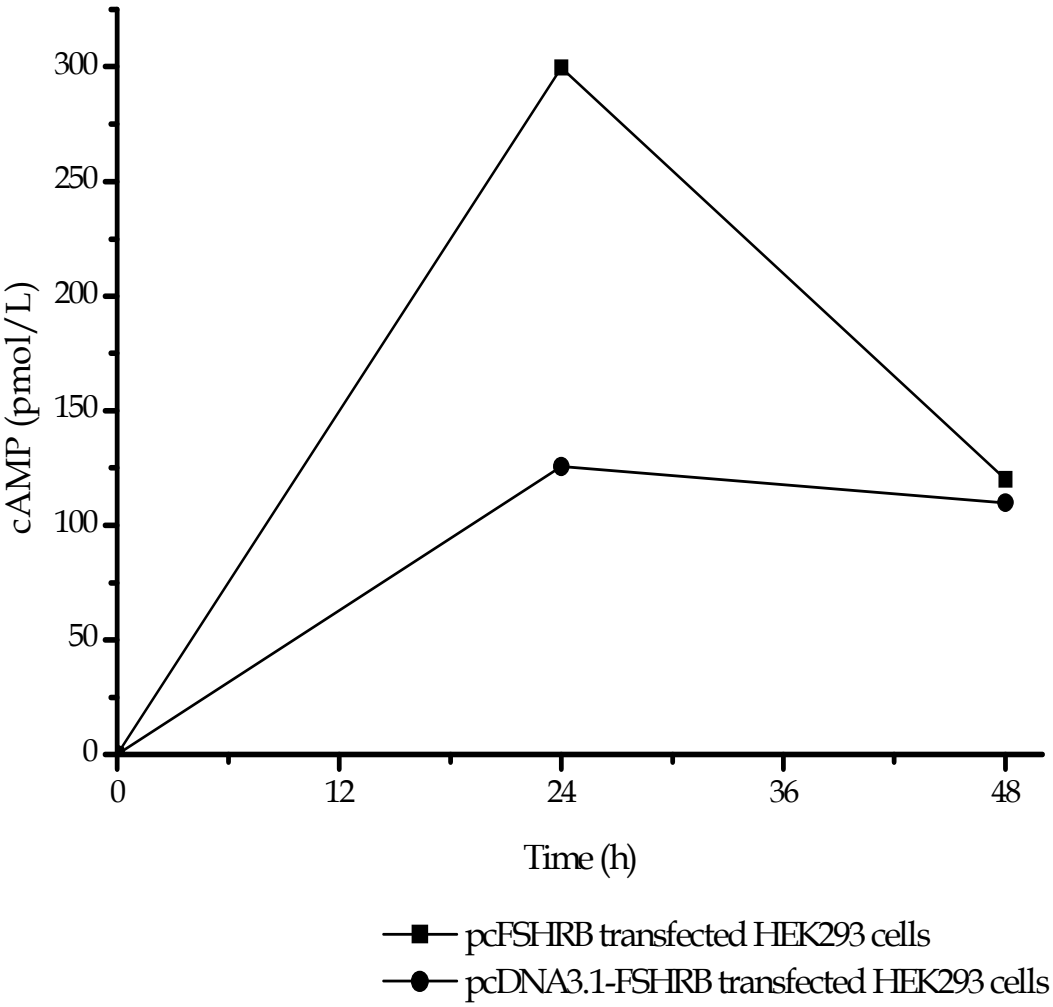

Figure 5. The production of cAMP in HEK293 cells. These cells were cultured for 24 h after transfection, the medium was changed with the new medium containing 2 IU/l FSH (Ningbo, China). After medium change, the cells were harvested at 24 and 48 h. The productions of cAMP were determined in these cells by RIA.

down. This may be caused by the FSHR desensitization, which has nothing to do with the promoter. (Manna et al., 1998)

DISCUSSION

Eukaryotic and prokaryotic transcription systems are quite different. In the prokaryotic cells, there is only one kind of promoter, but in the eukaryotic cells, there are 3 kinds of promoters. Type I and type III promoters are very simple and more similar to prokaryotic promoters. Type II promoters are more complex and contain many regulatory elements whose structures and localization are irregular (Wu, 2001). The FSHR promoter of the Jintang black goat is a typical eukaryotic type II promoter even though the sequences and position of TATA-box, CCAAT-box and GC-box are not typical and has some other typical *cis*-acting elements, such as GATA-box, E-box, USF, etc.

In the eukaryotes, the promoter always locates at the 5'-upstream of the corresponding gene. In the promoter, there are 1 TATA-box which is involved in the process of transcription by RNA polymerase located at -20 to -30 bp, 1 CCAAT-box which is related to the transcription efficiency located at -80 to -220 bp of the initial transcription site, 2 GC-box located at both sides of CCAAT-box (Wu, 2001). But in the FSHR promoter of the Jintang black goat, there is 1 non-typical TATA-box located at -92 bp from the initial transcription site, 1 non-typical CCAAT-box located at -210 bp, 1 non-typical GC-box located at -165 bp. The TATA-box is far away from the initial transcription site and might cause the initial transcription time delay. The non-typical CCAAT-box and GC-box located at -210 and -165 bp, respectively, are also far from the initial transcription site and might decrease the FSHR transcription efficiency. However, the

2 GATA-box in the promoter region could enhance the FSHR transcription efficiency (Kim and Griswold, 2001; Choudhury et al., 2008); the 3 A/T rich regions, which are conducive to the solution of double-stranded DNA helix, could improve the efficiency (Li et al., 2009); 1 USF which binds to E-box could enhance the efficiency; 1 E-box which binds to the bHLH transcription factors, also could enhance the efficiency of gene transcription (Heckert et al., 1998; Hermann et al., 2007).

Because of the large differences among the different species' genome, the FSHR promoter's nucleotides homology between the Jintang black goat and the *Gallus* is 32.88%, whereas that between the Jintang black goat and the mouse is 38.55%. In the same species, there are only 5 nucleotides difference, the homology is 99.34%. This indicates that the difference of FSHR promoter in different species is large and little in same species. This is consistent with those reported (Horikawa et al., 2005; Chabot et al., 2007).

The results showed that the FSHR promoter of the Jintang black goat can promote the FSHR gene strongly, but promote the EGFP gene weakly in HEK293 cells. This may be related to the gene difference, in that the FSHR promoter is the gene-special promoter. In the encoding region of FSHR gene, there may be some *trans*-acting elements which enhance the FSHR transcription efficiency.

Collectively, these results strongly suggest that the FSHR promoter of the Jintang black goat is a gene-special strong promoter and the gene has some gene-special transcription factor binding site regulating the transcription of FSHR promoter. Determining these factors will contribute to unveil the positive transcriptional regulation of this particular receptor promoter, which until now has remained elusive.

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