



Characterization of Gene Encoding Type-I Gonadotropin Releasing Hormone Receptor in Goat (*Capra hircus*)

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Abstract

In the present study, complete ORF of GnRH receptor transcript from goat pituitary was amplified, cloned in prokaryotic system and sequenced. The goat type-I GnRH receptor sequence shows high homology with sheep, cow, buffalo, equine and human. Like in other mammalian species in goat too, the GnRH receptor lacks a C terminal tail. Amino acids which are critical for structure and function of the receptor were found conserved. Few amino acid residues conserved amongst ruminants were found to be replaced in other mammalian species suggesting divergent evolutionary path of this gene in these groups.

Keywords: Gonadotropin releasing hormone receptor, *Capra hircus*, Pituitary, Accession number EF150356 and EU596505

1. Introduction

Gonadotropin Releasing Hormone (GnRH) is a key neuroendocrine regulator of the hypothalamic-pituitary-gonadal axis and plays an important role in mammalian reproduction by regulating synthesis and release of gonadotropins. GnRH is synthesized in hypothalamus and binds to a specific receptor called gonadotropin releasing hormone receptor (GnRH-R) located on plasma membrane of gonadotroph cells of anterior pituitary. In non mammalian vertebrates several subtypes of GnRH and its receptor are known. In mammals two subtypes of this hormone called GnRH-I and GnRH-II are found, but the only functional subtype of GnRH receptor found in mammals is type-I GnRH-R as in no species a type-II GnRH-R gene lacking a frame shift mutation is reported so far (Millar et al., 2004). Type-I GnRH-R is a member of seven transmembrane domain G protein coupled receptor super-family and is hypothesized to couple to

multiple G proteins (Conn and Croeley 1994). Unlike other members of this super-family, type-I GnRH-receptor lacks the carboxy terminal tail (Stojilkovic et al., 1994; Sealfon et al., 1997) which participates in several downstream reactions through several receptor-associated proteins (Bockaert et al 2003; Ferguson et al., 2001). GnRH receptor transcripts have also been detected in some extrapituitary tissues, including ovary (Choi et al., 2001; Kang et al., 2000 and 2001) and placenta (Siler-Khodr et al., 2001; Khodr et al., 1980; Chou et al., 2004) and there are increasing evidences that GnRH may act as an autocrine or paracrine factor in regulating local cellular functions in these organs. In the ovary GnRH modulates both basal and gonadotropin-stimulated steroidogenesis (Olofsson et al., 1995) and induces transcription of several genes involved in the follicular maturation process and ovulation. High-affinity binding sites for GnRH-I have been reported in human corpus luteum, luteinized granulose cells, and a number of ovarian cancer cell lines (Bramley et al., 1987; Emons et al., 1989 and 1993; Brus et al., 1997). Though goat is an economically important animal for Asian countries, particularly India, no information is available on GnRH receptor of goat. In the present study, transcript of type-I GnRH receptor has been characterized in goat pituitary. The goat type-I GnRH receptor sequence shows high homology with sheep, cow, buffalo, equine and human. Like in other mammalian species in goat too, the GnRH receptor lacks a C terminal tail. Amino acids which are critical for structure and function of receptor were found conserved. Few amino acid residues conserved amongst ruminants were found to be replaced in other mammalian species suggesting divergent evolutionary path of this gene in these groups.

2. Materials and methods

2.1 Total RNA isolation

For total RNA isolation, pituitaries were collected from local abattoir, immediately after slaughter in RNAlater solution (Qiagen, Germany). Total RNA isolation was done using RNeasy Mini kit (Qiagen, Germany) according to manufacturer's protocol. Total RNA was quantified and its integrity was assessed using 1% formaldehyde agarose gel electrophoresis.

2.2 cDNA synthesis

For cDNA synthesis 5µg of total RNA was reverse transcribed into first strand cDNA, using oligodeoxythymidine primers (MBI-Fermentas). Each reaction included 1 X RT buffer 50 mM Tris-HCl (pH 8.3), 50mM KCl, 4mM MgCl₂, 5mM DTT (MBI-Fermentas), 2.5 mM of each of the four deoxy-NTPs, 0.5µl oligo-dT primers, 20 U Ribonuclease inhibitor, and 200 U Moloney murine leukemia virus reverse transcriptase (MBI-Fermentas) and was carried out at a volume of 25 µl for 1 h at 37°C. Reaction was stopped by heating at 70°C. A parallel control reaction was carried out without adding reverse transcriptase. After the end of the reaction, the volume was increased to 100µl with ultrapure water, and the mixture was stored at -20°C.

2.3 PCR amplification

The primers were designed to amplify two PCR products a 408 bp product and a 1026 bp product on the basis of the available bovine and ovine GnRH receptor sequences. Primers used to amplify 1026 bp product were, forward 5'ATAAAGGATGGCAAACAGTGAC3', reverse 5'GCCTTCTTTGACTTTCTATGC3' and primers for 408 bp product were forward 5'CCT CCC CAC CCT GAC CCT ATC T-3' and reverse 5'GCCAAGCCAATCATGAACTGTCC3'. Standard protocol according to Sambrook and Russel (2001) was used for PCR amplification. Reaction was carried out in 25 µl reaction volume containing 70 ng of cDNA, 10 pM of each primer, 400µM of dNTP mix, 2.5 unit of Taq DNA polymerase and 1.5mm of MgCl₂ using T personal thermocycler. The PCR programme used to amplify 408 bp product involved initial denaturation at 95°C for 3 min followed by 32 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 1 min ending with a final extension at 72°C for 10 min. Same reaction volumes, conditions of temperature and time were used to amplify 1026 bp product, except that annealing temperature was 48°C. The amplified PCR products were electrophorized in with 100 bp DNA ladder (MBI Fermentas) on ethidium bromide stained 1% agarose gel and were visualized and photographed under UV.

2.4 Cloning and sequencing of PCR product

Both 1026 and 408 bp PCR products were cloned in prokaryotic system and sequenced. For this PCR products were purified using gel extraction kit (Qiagen) following manufacturers protocol. The purified PCR product was ligated to pTZ57R/T (MBI Fermentas) cloning vector using 50 ng purified PCR products 50ng of vector, and 5U of T₄ DNA Ligase. The reaction mixture was incubated at 22°C overnight in water bath. The transformation of DH5α E.Coli cells with ligated product was done using Insta cloning kit (MBI Fermentas) following manufactures procedure. The transformed cells were spread over ampicillin agar plate coated with 40 µl of 0.1 m IPTG and 40 µl of 20mg/ml X Gal. The white colonies were propagated in 3 ml LB broth containing ampicillin by overnight incubation in a shaker incubator at 37°C. Presence of insert was confirmed by plasmid isolation using alkaline lyses method followed by restriction digestion with *EcoRI* and *SalI* enzymes. For sequencing cloned PCR products, DNA sequencing facility at University of Delhi South Campus (UDSC), New Delhi was availed.

3. Results

3.1 RNA isolation and RT-PCR

The RNA yield using RNAeasy mini kit was found 1.5 µg/mg of tissue and 260/280 of the RNA sample obtained was 1.98 indicating the high purity of the RNA sample. (Fig. 1) Primers used for the amplification were designed on the basis of published sequences of bovine and sheep GnRH-R gene. PCR with these primers gave products of expected size i.e. 1026 bp (Fig. 2) and 408 bp products (Fig. 3) from pituitary.

3.2 Cloning and Sequencing of PCR products

About 110 colonies were observed on the LB plates. Colonies were screened by blue and white selection method. Most colonies were white and few blue. The white colonies were propagated for the plasmid isolation and further confirmation. Alkaline lysis method was found to be an easy and fast method for plasmid isolation. Isolated plasmid was free from genomic DNA and RNA contamination. The yield of plasmid was 484 ng/µl and has shown a 260/280 ratio of 1.8 which was sufficient for carrying out subsequent screening steps. Inst/A cloning vector has restriction sites for *EcoRI* and *SalI*, which were absent in the insert. Therefore for screening of recombinant clones, plasmids isolated from different white colonies were subjected to double digestion with restriction enzymes, *EcoRI* and *SalI*. Insert release of about 1026 bp and 408 bp were confirmed by agarose gel electrophoresis. Clones confirmed for the presence of desired fragments were sequenced at UDSE New Delhi. The obtained sequences were analyzed using DNASTAR software. The sequence homology was confirmed using Basic Local Alignment Search Tool (BLAST) programme of National Centre For Biotechnology Information Gene Bank (www.ncbi.nlm.nih.gov). Sequences were submitted to Gene Bank NCBI and Accession number EF 150356 and EU 516505 were obtained.

4. Discussion

The amplified 1026 bp product spans complete ORF, which is 987 bp long, position 8 to 994 (Fig. 5) The 408 bp product corresponds to position 81 to 488 of the ORF. The 408 bp product resides completely within the Exon-I region and thus can be used as a probe for type-I GnRH-R gene identification in southern dot blot analysis with RT-PCR product as well as with genomic DNA. The ORF of goat type I GnRH was aligned with that of other mammalian species using MegAlign programme of DNASTAR software. The goat type-I GnRH ORF shows more than 85% homology with different animal species. The sequence shows high homology with GnRH receptor sequences of sheep (99%), cow (98%), buffalo (98.4%), equine (91.5%) and human (88.4%). The phylogenetic tree of type-I GnRH-R gene of different mammalian species show different evolutionary pattern as GnRH-R gene of goat and sheep fall in one group which in turn share close evolutionary resemblance with primate and distinct relation with rodents (Fig. 4). The amino acid sequence of goat type-I GnRH-R bears all the characteristic features for transmembrane receptor. High numbers of hydrophobic amino acid residues (141 out of 328) in deduced amino acid sequence were found, which is a characteristic feature of transmembrane protein. The length of the protein suggests that like other mammalian species in goat too, the GnRH-R lacks a C terminal tail. The deduced amino acid sequence was aligned with that of other species using MegAlign programme (Fig. 6). Upon alignment of the amino acid sequence of type-I GnRH receptor of several mammalian species it was found to be more than 90% conserved. Goat type I GnRH-R share close similarity with sheep (98.5%), buffalo and cow (97.9%) and equine (92.7%), lower similarity with human and macaca (89.7% and 89.9% respectively) and lowest similarity with mouse and rat (86.0% and 84.8%). These results are in accordance with the nucleotide sequence analysis. The putative transmembrane domains were found almost identical with in these mammalian species. Amino acid residues important for receptor function for instance, Ala (261) in the third intracellular loop which is crucial for G protein coupling and receptor internalization (Myburgh et al., 1998), Asp (98), Trp (101) Asn (102), Lys (121), Asn (212) and asp (302) which are important for ligand binding (Hoffmann et al., 2000) were found conserved in all species. The GnRH receptor was found conserved amongst mammalian species but species wise difference were found at several positions, for example, Ser (27) is well conserved amongst ruminants (cow, buffalo, sheep and goat), but is replaced by Lys in rodents (mouse and rat) and by Asn in human and macaca monkey, residue Ile (51) which is conserved amongst ruminants is replaced by Ala in rodents and Thr in human and macacca. Several such species wise variations were observed in amino acid sequences of Type-I GnRH-Receptor in different mammalian species. These results suggest the divergent evolutionary path of Type-I GnRH receptor in different animal groups. Type-I GnRH-R appears to have undergone a relatively recent period of rapidly accelerated molecular evolution (Fig 4) that may have been important for the development of mammalian reproductive strategies. The present study provide the necessary information about Type-I GnRH-R in goat and paves the way for further molecular work related to this receptor in goat, like PCR based confirmation of expression of this receptor in different tissues and quantitative PCR (real time PCR) to study different expression levels of this receptor.

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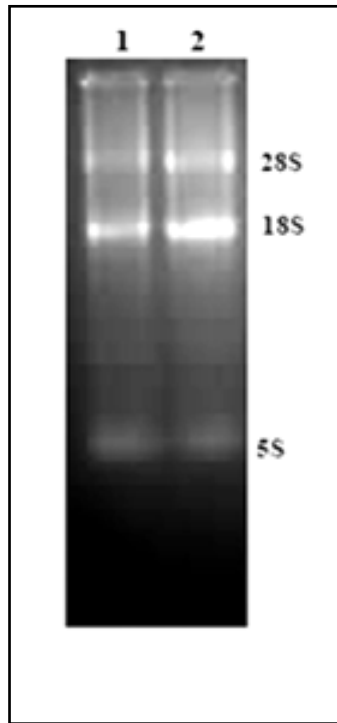


Figure 1. Formaldehyde Agarose Gel electrophoresis of Total cellular RNA from Goat Pituitary

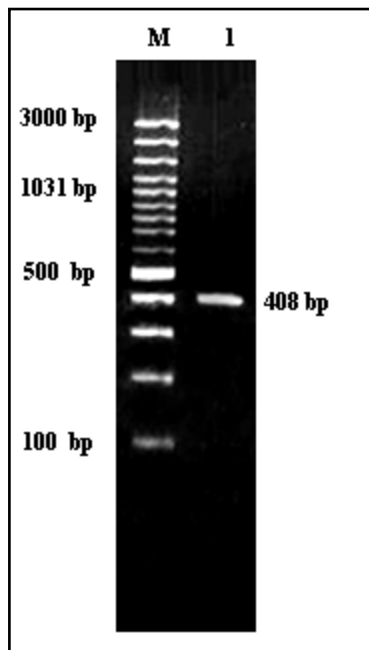


Figure 2. Agarose Gel Electrophoresis of 408 bp purified PCR Product from goat pituitary cDNA. Lane M Molecular size marker, Lane 1 purified 408 bp product

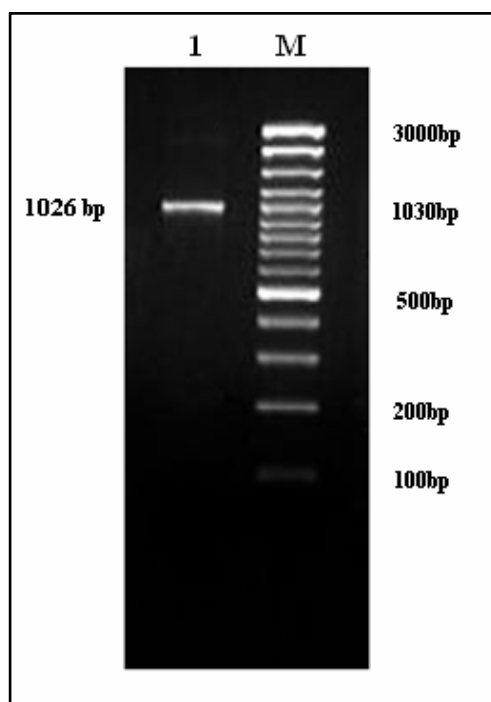


Figure 3. Agarose gel electrophoresis of purified 1026bp PCR product Lane M Molecular size marker, Lane 1 purified 1026 bp purified product

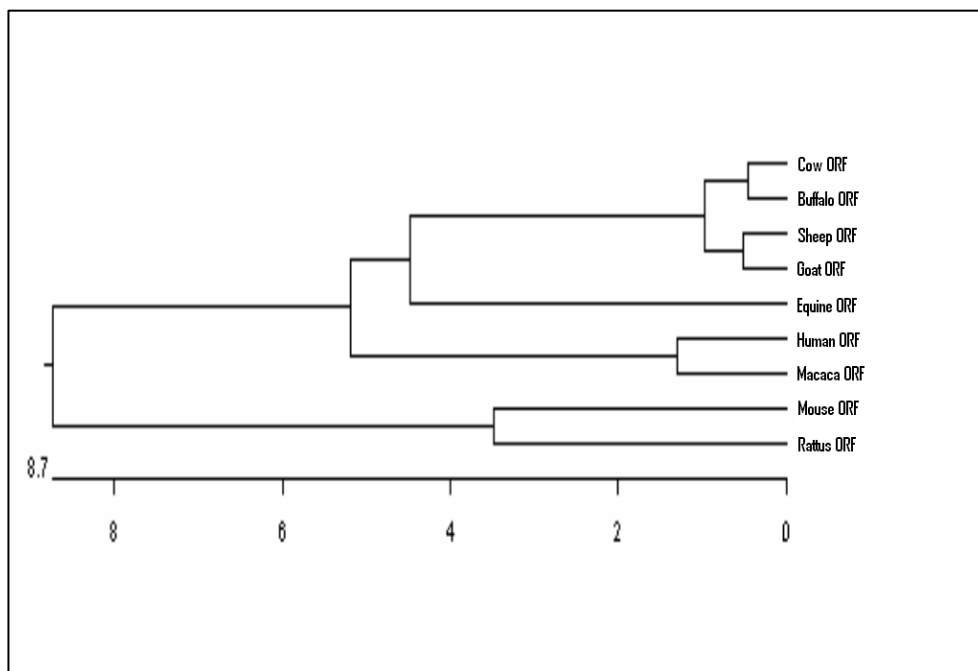


Figure 4. Phylogenetic tree of Type-I GnRH Receptor gene based on the sequences of different animal species

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8 atggcaaacagtgactctcctgaacagaatgaaaaccactgttca  
53 gcaatcaacagcaacatcctactaacaccgggcagcctcccacc  
98 ctgacccctatctgaaaagatccgagtgacagttactttcttctt  
143 tttctactctccacaattttcaacacttcttcttgttgaactt  
188 cagaaattgactcagaggaagacaagacgaaaaactctcaaaa  
233 atgaaggtgcttttaaaacactgactttaagccaacctgctggag  
278 actctgattgtttatgccactggtatggaatgtggaatataactggt  
323 caatggtatgctggagagctcctttgcaaaagtctcagctatctg  
368 aagcttttctccatgtacgcccccgccttcctgatggtggtgatc  
413 agcctcgaaccgctccctggccatcaccagcctctagcagtgaaa  
458 agcaacagcaagctcggacagttcatgattggcttggcctggctc  
503 ctcagtagcatctttgctggaccacagttatacatctttgggatg  
548 atccatttagcagatgactctggacagactgaaggtttctctcaa  
593 tgtctaacacactgcagttttccacagtggtggcatcaagccttt  
638 tataaacttttcaacttcagctgcctcttcatcatccctcttctc  
683 atcatgctgatctgcaatgcaaaaatcatcttcaccctaaacagt  
728 gtccttcatcaggatccccacaaactacaactgaatcagtccaag  
773 aacaatataccacagactcggctgaggraccctaaagatgacggtt  
818 gcatttgccacttcatttactgtctgctggacgccctactatgtc  
863 cttggaatttggtatgggtttgatcctgacatggtaaacaggtg  
908 tcagatccagtaaatcaactcttcttctctttgcttttttaaat  
953 ccatgctttgatccacttatatggatatttctctctataa 994
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Figure 5. ORF of goat type-I GnRH-R gene

M A N S A S P E Q M E M H C S A I N S S I P L T Q G S L P T L T L S G K I R V T		Majority
	10 20 30 40	
1 D L P	GOAT
1 D P	BUFFALO
1 D P	COW
1 Q S A N	EQUINE
1 Q N M N	HUMAN
1 L V N M N	MACACA
1 N L D P N I K V	MOUSE
1 G D D L P	SHEEP
1 N L D Q N K	RAT
V T F F L F L L S T I F N A S F L L K L Q N W T Q K X E K G K K L S R M K V L L		Majority
	50 60 70 80	
41 T R R K	GOAT
41 T R R L	BUFFALO
41 T R R L	COW
41 T H K N K	EQUINE
41 & T K L	HUMAN
41 & T K L	MACACA
41 & K R K	MOUSE
41 T R R K	SHEEP
41 & V R R K	RAT
X H L T L A M L L E T L I V M P L D G M W N I T V Q W Y A G E L L C K V L S Y L		Majority
	90 100 110 120	
81 F	GOAT
81 F	BUFFALO
81 F	COW
81 F	EQUINE
81 F	HUMAN
81 F	MACACA
81 F	MOUSE
81 F	SHEEP
81 F	RAT
K L F S M Y A P A F M M V V I S L D R S L A I T R P L A V K S N S K L G Q S M I		Majority
	130 140 150 160	
121 F	GOAT
121 K F	BUFFALO
121 K F	COW
121 R	EQUINE
121 L V V	HUMAN
121 L S V	MACACA
121 Q Q E	MOUSE
121 F	SHEEP
121 V Q Q K E R T	RAT
G L A W L L S S I F A G P Q L Y I F R M I H L A D D S G Q T E G F S Q C V T H C		Majority
	170 180 190 200	
161 G	GOAT
161 G	BUFFALO
161 G	COW
161 S	EQUINE
161 I V S K V	HUMAN
161 I V S K V	MACACA
161 S I I V Y G P - V	MOUSE
161 G	SHEEP
161 S I I V Y G P A - V	RAT
S F P Q W W H Q A F Y N F F T F S C L F I I P L L I M L I C N A K I I F T L T R		Majority
	210 220 230 240	
201 S	GOAT
201 V	BUFFALO
201 V	COW
201 F	EQUINE
201 S F	HUMAN
201 G	MACACA
200 A	MOUSE
201 A	SHEEP
200 E	RAT

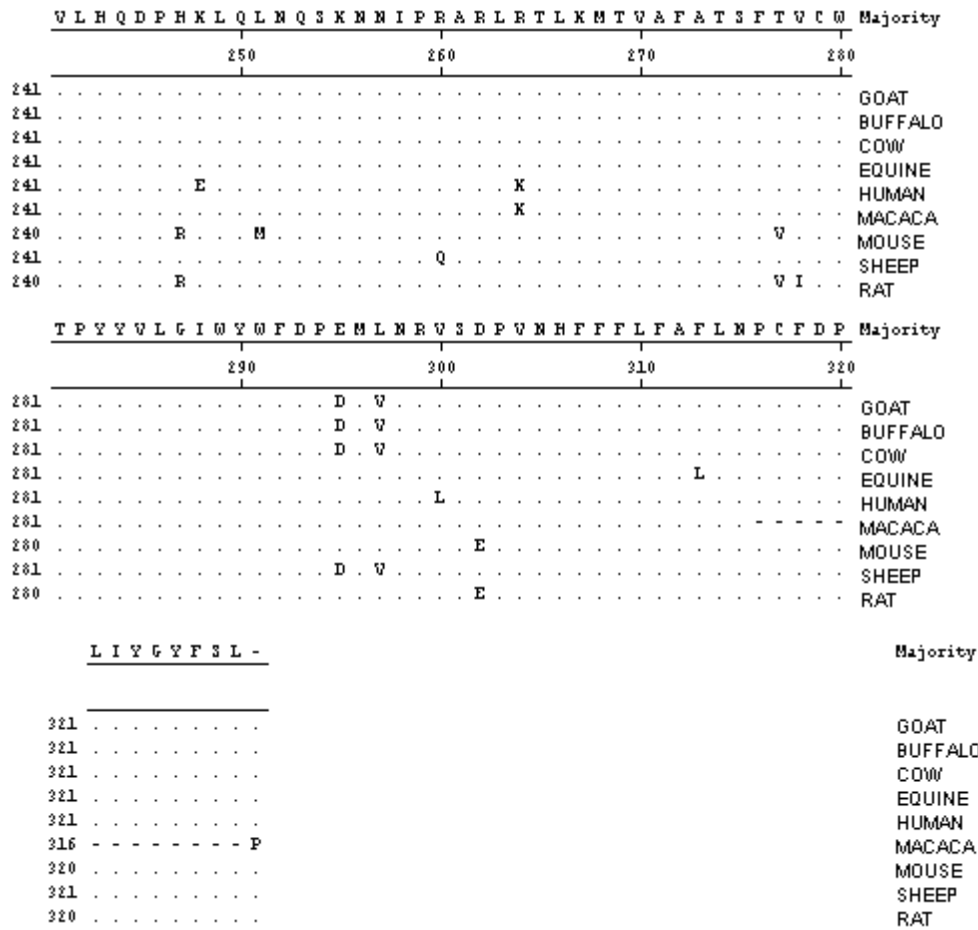


Figure 6. Alignment of goat type-I GnRH-R deduced amino acid sequence with other animals