# Functional Activity of the Porcine Type II GnRH Receptor Gene Promoter

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## Abstract

Binding of GnRH to its receptor represents a central point for regulation of reproduction in mammals. Recently, a different mammalian GnRH decapeptide has been identified, GnRH-II, differing from the classical peptide, GnRH-I, by three amino acids. Consistent with this, GnRH-II elicits its effects via a new receptor isoform, type II GnRH receptor (GnRHRII). The GnRH-II system may play a role in puberty attainment, sexual behavior, food intake and energy balance. Despite the presence of a functional GnRHRII in some mammals, the GnRHRII gene is absent in mice and silenced or disrupted in the human, chimpanzee, sheep, cow and rat. In contrast, the porcine GnRHRII may be functional as no gene disruptions have been detected. To examine its functionality, we isolated the 5' flanking sequence for the porcine GnRHRII gene. A porcine genomic BAC library was screened for the GnRHRII gene using probes designed from the previously reported porcine GnRHRII cDNA sequence. The BAC clone DNA was digested and the resulting fragments were self-ligated, forming multiple circular DNA fragments of the BAC DNA. Inverse PCR was performed on these circular DNA fragments utilizing primers developed from the published porcine GnRHRII cDNA sequence that amplify in opposite directions. After sequencing the resultant PCR product, a BLAST search comparing this sequence with the porcine genome identified approximately 3,000 bp of 5' flanking region for the porcine GnRHRII gene. Next, we constructed a vector containing 3,000 bp of the porcine GnRHRII promoter fused to the cDNA encoding luciferase (GnRHRIIpGL3). To examine functionality of the porcine GnRHRII promoter, transient transfections were performed in gonadotrope-derived alphaT3-1 cells with GnRHRIIpGL3, a vector containing the promoter for the type I GnRHR (GnRHRIpGL3) and a promoterless control. Our laboratory has established robust activity of the GnRHRIpGL3 vector in this cell line. Luciferase activity for cells transiently transfected with GnRHRIIpGL3 and GnRHRIpGL3 was 12- and 10-fold above promoterless controls, respectively. In other mammalian species, mRNA for the type II GnRHR has been detected in many other tissues. To determine if the porcine GnRHRII promoter was functional in non-gonadotrope cell lines, we transiently transfected GnRHRIIpGL3, GnRHRIpGL3, a promoterless control (pGL3) and RSVpGL3, as a positive control, into porcine kidney- (PK-15), granulosa cell- (PGC2) and testis-derived (ST) cell lines. Results indicated that GnRHRIIpGL3 activity was significantly higher than promoterless control in PK-15 (12-fold), PGC2 (12-fold) and ST (11-fold) cells. Therefore, approximately 3,000 bp of the porcine GnRHRII gene promoter is functional in gonadotrope-derived alphaT3-1 cells. However, in contrast to the promoter for the porcine type I GnRHR, the GnRHRII promoter is also active in porcine granulosa cell-, testis-, and kidney-derived cells. Thus, the pig may serve as a unique model for investigation of the GnRHRII gene.

## Introduction

To date, three isoforms of GnRH have been identified, GnRH-I, -II, and -III (Table 1). GnRH-I is the original decapeptide, which is known to play a critical role in the regulation of reproduction. A second decapeptide that closely resembles GnRH-I was first isolated from the chicken and since has been found in many species, from bony fish to humans. GnRH-III has been discovered in lamprey fish, but has not been studied as thoroughly as the other isoforms.

Table 1. Amino acid sequences of GnRH isoforms.

| GnRH Isoform | Amino Acid Sequence |
|---|---|
| GnRH-I | pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2 |
| GnRH-II | pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2 |
| GnRH-III | pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH2 |

GnRH-I is released in a pulsatile nature from the hypothalamus and travels to the anterior pituitary gland where it binds to its specific receptor, GnRHR-I, on the plasma membrane of gonadotrope cells. Binding of GnRH-I to its receptor induces the synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Stimulation of the GnRHR-I by GnRH-I induces up-regulation of genes encoding the common alpha-subunit, the unique beta-subunits specific to LH and FSH, and GnRHR itself.

A type II GnRH receptor has recently been identified that has a high affinity for GnRH-II, however functional GnRHR-II protein is not present in many mammalian species. Consistent with this, a disrupted gene sequence has been found in the rat, sheep, cow, and human. Also, mice do not contain any DNA sequence that resembles the GnRHR-II gene. In contrast, a functional receptor has been found in musk shrews and old-world monkeys. Studies by Kauffman *et al.* (2, 3) in musk shrews suggested that this receptor is involved in female mating behavior and energy balance. Based on DNA sequence, pigs retain the potential to produce a functional GnRHR-II. Neill *et al.* (1) identified coding sequence for two type II GnRH receptor isoforms in the pig, a classic 7 transmembrane, G-protein coupled receptor and a receptor lacking the first two transmembrane domains. Therefore, our studies were designed to examine functionality of the porcine GnRHR-II.

Table 2. Species distribution of GnRH and GnRHR isoforms (Adapted from Millar, 2003)

| Animal | GnRH I | | GnRH II | | GnRH III | |
|---|---|---|---|---|---|---|
| | Ligand | Receptor | Ligand | Receptor | Ligand | Receptor |
| Fish | + | + | + | - | + | + |
| Amphibians | + | + | + | + | + | + |
| Reptiles | + | + | + | + | - | - |
| Birds | + | + | + | ? | - | - |
| Pig, Monkey | + | + | + | + | - | - |
| Cow, Sheep, Chimpanzee, Human | + | + | + | - | - | - |
| Mouse | + | + | - | - | - | - |

## Objectives

1. Isolate the porcine type II GnRHR gene promoter.
2. Determine functionality of the type II GnRHR promoter in gonadotrope- and non-gonadotrope-derived cells.
3. Compare the activity of the type I and II GnRHR promoters in gonadotrope- and non-gonadotrope-derived cells.
4. Identify minimal regions of the type II GnRHR promoter important for activity.

## Results

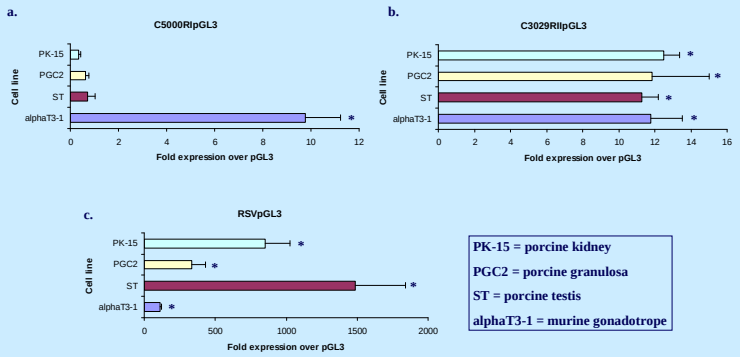


**Figure 1. Functional activity of the (a) type I GnRHR promoter (C5000RIpGL3), (b) type II GnRHR promoter (C3029RIIpGL3), and (c) a constitutively active promoter (RSVpGL3) in gonadotrope- and non-gonadotrope-derived cell lines.** Asterisks indicate normalized luciferase values that are higher than promoterless control ($P < 0.05$).

## Materials and Methods

- A porcine BAC library was probed for the type II GnRH receptor.
- Inverse PCR was performed on the DNA isolated from positive BAC clones.
- Approximately 4,000 bp of the 5' flanking sequence was identified from the resulting DNA fragments.
- A BLAST search using the published cDNA sequence within the porcine genome detected three exons and two introns for the type II GnRHR gene.
- 3029 bp of the type II GnRHR promoter was subcloned into a luciferase reporter vector (C3029RIIpGL3).
- Transient transfections were performed with C3029RIIpGL3; a vector containing the type I GnRHR promoter, C5000RIpGL3; a vector containing a constitutively active promoter, RSVpGL3; and a promoterless control (pGL3) using a liposome-mediated protocol (Fugene6, Roche Molecular). RSV-beta-galactosidase was cotransfected with each reporter vector.
- Three replications were completed in triplicate using DNA from at least three different plasmid preparations.
- The transfected cells were harvested and luciferase (Promega) and beta-galactosidase (Tropix) assays were performed on the cellular extracts.
- To normalize for transfection efficiency, the luciferase values were divided by beta-galactosidase values. Means are expressed as fold changes over the promoterless control.
- Transfected cell lines were PK-15, a porcine kidney-derived cell line; PGC2, a porcine granulosa cell-derived cell line; ST, a swine testis-derived cell line; and alphaT3-1, a murine gonadotrope-derived cell line.
- A deletion construct (C2355RIIpGL3) was then produced and transiently transfected into the alphaT3-1 cell line with C3029RIIpGL3 and pGL3.

## Results

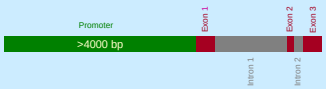


**Figure 2. Depiction of the porcine type II GnRHR gene including the promoter region, three exons and two introns.**

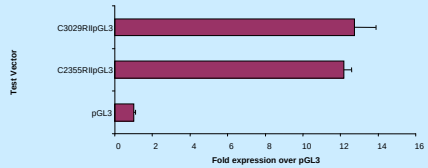


**Figure 3. Reduction of the porcine GnRHR-II promoter from 3029 to 2355 bp maintained luciferase activity.**

## Conclusions

Type II GnRHR promoter activity is distinct from the type I GnRHR promoter. The type I GnRHR promoter is active in gonadotrope-derived alphaT3-1 cells alone, whereas the type II GnRHR is active in cells of both gonadotrope and non-gonadotrope origins. This suggests, not only ubiquitous expression of the GnRHR-II gene, but also a non-reproductive function for the GnRH II system. Finally, activity of the type II GnRHR promoter is conferred by 2355 bp of 5' flanking region.

## Future Aims

1. Perform transient transfections in additional cell lines.
2. Identify the minimal 5' flanking region required for promoter activity in alphaT3-1 cells.
3. Analyze expression patterns of the type II GnRHR gene in porcine tissues by real-time PCR.

References:

1. Neill *et al.* 2004. TRENDS in Endocrinology and Metabolism. 15:383-392.
2. Kauffman *et al.* 2006. Endocrinology. 147:5069-5077.
3. Kauffman *et al.* 2005. Journal of Neuroendocrinology. 17:489-497.
4. Millar. 2003. TRENDS in Endocrinology and Metabolism. 14:35-43.