Transcriptional Regulation of the Porcine GnRH Receptor (GnRHR) Gene

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Abstract

Physiologically, the interaction of GnRH with its cognate receptor represents a central point for regulation of reproductive function. In the pig, the GnRHR gene is also a positional candidate for genes critical to reproductive efficiency. Both the GnRHR gene and a quantitative trait locus (QTL) for ovulation rate have been mapped to the same region of porcine chromosome 8. Following initial isolation of the genomic sequence for the porcine GnRHR gene, we utilized inverse PCR to isolate approximately 5000 bp of 5' flanking sequence. We have developed a model system to identify elements that confer cell-specific expression of the porcine GnRHR gene in gonadotrope-derived αT3-1 cells. Constructs containing either 1800 or 1000 bp of porcine promoter or 600 bp of murine promoter fused to the cDNA encoding luciferase were produced (-1800pGL3, -1000pGL3 and murine -600pGL3, respectively). To test functionality of these vectors, liposome-mediated transient transfection assays were performed in αT3-1 cells. Following a 24 h transfection, cells were harvested and assayed for luciferase and β-galactosidase activity. Values for luciferase activity were adjusted for β-galactosidase and expressed as fold changes over promoterless controls. Luciferase values for the murine -600pGL3 vector were significantly higher than either of the porcine promoters (24-fold). However, expression levels for all vectors were significantly higher than promoterless controls. Strikingly, reduction of the porcine proximal promoter from 1800 to 1000 bp resulted in a dramatic loss of promoter function (12- vs. 3-fold). Sequence analysis of this region has identified a number of putative elements, including a consensus activator protein-1 element. Thus, initial studies with the porcine GnRHR gene promoter have revealed that a key element(s) required for basal expression may be present between -1800 to -1000 bp of the translational start site.

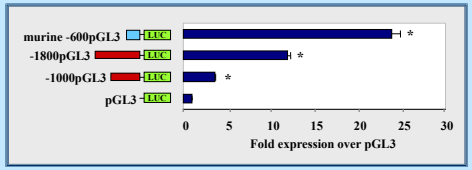

Figure 1. Preliminary transient transfection of αT3-1 cells with luciferase vectors (pGL3) containing the -1800 and -1000 porcine promoters and the murine -600 promoter. Bars with an asterisk are greater than pGL3 ($P < 0.05$)

Introduction

The genomic sequence for the porcine GnRHR gene has only recently been identified leading to isolation of a portion of 5' flanking sequence (~1100 bp) for this gene. This discovery has opened a new arena of research and will greatly enhance our abilities to improve reproductive efficiency of swine. The interaction of GnRH with its receptor is well established as a pivotal point in regulation of normal reproductive function. GnRH is secreted from the hypothalamus and travels through the hypothalamic-hypophyseal portal system to the anterior pituitary gland where it binds its cognate receptor on gonadotrope cells. The binding of GnRH to its receptor results in the synthesis and secretion of luteinizing hormone and follicle stimulating hormone, as well as up-regulation of GnRHR. In addition to the physiological role of the receptor, the GnRHR gene itself is located in the same region of porcine chromosome 8 as a quantitative trait locus (QTL) for ovulation rate. Thus, the GnRHR gene is a positional candidate for genes critical to reproductive efficiency including ovulation rate. We are interested in further eliciting the role of the GnRHR gene in reproductive function of swine and the possible implications for enhancement of pork production. Towards this end, we must first identify the molecular mechanisms underlying transcriptional regulation of the porcine GnRHR gene.

Objectives

1. Sequence additional 5' flanking region of the porcine GnRHR gene.
2. Develop an *in vitro* model system to study expression of the porcine GnRHR gene.
3. Utilize luciferase reporter assays to determine promoter regions necessary for basal expression of the porcine promoter in gonadotropes.

Materials and Methods

Promoter Sequencing:

- Inverse PCR was utilized to generate additional 5' flanking sequence of the porcine GnRHR gene.

Reporter Constructs:

- The full-length promoter (-5000) was amplified by PCR and subcloned into a luciferase reporter vector (pGL3, Promega).
- A series of 5' deletion constructs were created from -5000pGL3 using restriction enzyme digests.

Transfections:

- The murine gonadotrope-derived cell line, αT3-1, was maintained in DMEM with 5% FBS, 5% horse serum, 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM glutamine at 37°C in a humidified 5% CO_2 in air environment.
- Two million cells were plated in individual wells of a 6-well plate the afternoon prior to transfection.
- Transient transfections were performed using Fugene6 (Roche Molecular) according to manufacturer's instructions. The reporter vector (0.9 μg) was cotransfected with a control vector, RSV-β-galactosidase (0.1 μg).
- Three transfections were completed in triplicate using DNA from different plasmid preparations.

Luciferase and β-galactosidase Assays:

- Approximately 24 h post-transfection, cells were washed with ice-cold PBS, lysed using a commercial lysis buffer (Tropix) supplemented with 1 mM DTT, and harvested.
- Luciferase (Promega) and β-galactosidase (Tropix) assays were performed per manufacturer's instructions.
- Luminescence was detected with a Wallac Victor2 plate reader (PerkinElmer).
- Luciferase values were divided by β-galactosidase activity to normalize for transfection efficiency and means were expressed as fold changes over promoterless control (pGL3).
- Means for adjusted luciferase activity were analyzed using ANOVA and compared using either Dunnett's two-tailed *t*-test or Tukey's studentized range test.

Results

- In preliminary studies, luciferase reporter constructs containing either 1800 or 1000 bp of the porcine promoter (-1800pGL3 and -1000pGL3) were produced. Transfection results indicated a dramatic decrease (12-fold vs. 3-fold) in luciferase activity when the promoter is reduced from 1800 to 1000 bp (Figure 1). The 12-fold expression of -1800pGL3 in αT3-1 cells was higher than expression levels reported for the bovine, human, or ovine promoters. However, luciferase activity of -1800pGL3 was significantly lower than that of positive controls (murine -600pGL3), leading to future studies with additional 5' flanking sequence.
- Initial transfection studies in the αT3-1 cell line were performed to compare constructs containing either 5000 bp of the porcine or 600 bp of the murine GnRHR promoter fused to the cDNA encoding luciferase (-5000pGL3 and murine -600pGL3). The murine -600pGL3 vector is well-established as a positive control in αT3-1 cells. The robust luciferase levels achieved in cells transfected with the -5000pGL3 indicated that this *in vitro* model system provides the sensitivity needed for deletion and mutation studies of the porcine GnRHR promoter (Figure 2).
- To determine regions that are important for basal expression of the porcine GnRHR gene promoter, deletion constructs were used in transient transfection experiments with the αT3-1 cell line (Figure 3). Large decreases in luciferase activity between deletion constructs indicates that important elements are likely to be located in the following regions; -500pGL3 to -1000pGL3, -1400pGL3 to -1900pGL3, and -2300pGL3 to -3100pGL3.
- Also of interest, sequence analysis has identified several putative elements within the promoter including AP-1, AP-2, and SURG-1 elements, as well as binding sites for Pitx1, Pit-1, Sp1, and SF-1 (Figure 4).

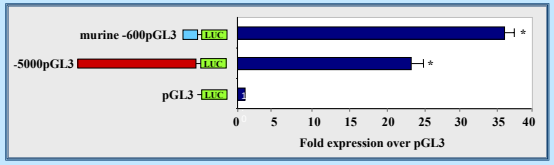

Figure 2. Transient transfection of αT3-1 cells with luciferase vectors (pGL3) containing the -5000 porcine promoter and the murine -600 promoter. Cells transfected with the -5000 promoter had levels of luciferase activity near that of the positive control. Bars with an asterisk are greater than pGL3 ($P < 0.05$)

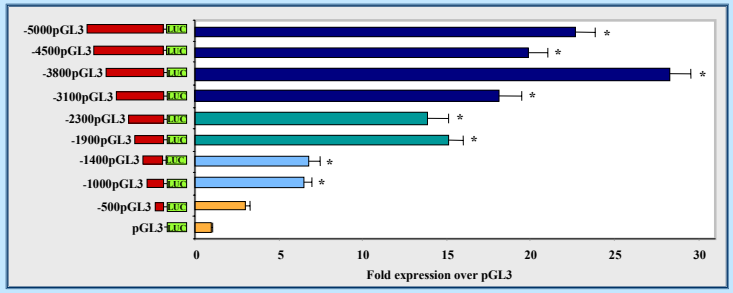

Figure 3. Transient transfection of αT3-1 cells with luciferase vectors (pGL3) containing the full-length porcine promoter (-5000pGL3) and sequential 5' deletions of -5000pGL3. Different bar colors highlight promoter regions with reduced luciferase activity ($P < 0.05$). These regions may contain elements important for basal expression of the porcine GnRHR gene. Bars with an asterisk are greater than pGL3 ($P < 0.05$)

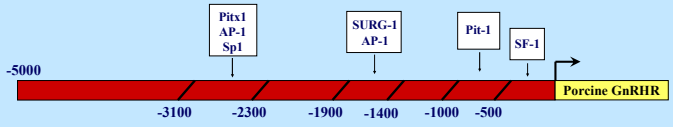

Figure 4. Diagram of a hypothetical cell-specific enhancer located within the 5' flanking region of the porcine GnRHR gene. These putative elements have been shown to contribute to cell-specific expression of gonadotropic genes from other mammalian species.

Future Aims

1. Generate mutation constructs based on results of deletion studies and sequence analysis.
2. Perform electrophoretic mobility shift assays to further evaluate the putative elements and transcription factors involved in transcriptional regulation of the porcine GnRHR gene.