Transcriptional Regulation of the Porcine GnRHR 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 gonadotrop-derived-differentiated T3-1 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, lipsomemediated transient transfection assays were performed in T3-1 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 activity to normalize for transfection efficiency and means were expressed as fold changes over promoterless controls (control = pGL3). Means for adjusted luciferase activity were analyzed using ANOVA and compared using either t-test or Tukey’s studentized range test. Initial transfection studies in the T3-1 cell line were used to compare constructs containing either 1800 bp of the porcine or 600 bp of the murine GnRHR promoter fused to the cDNA encoding luciferase (-1800pGL3 and -600pGL3). The murine-600pGL3 vector was well-established as a positive control in T3-1 cells. The robust luciferase levels achieved in cells transfected with the -5000pGL3 indicated that this in vivo model 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 T3-1 cell line (Figure 3). Large decreases in luciferase activity between deletion constructs indicate that important elements are likely to be located in the following regions: -5000pGL3 to -1000pGL3, -1000pGL3 to -500pGL3, and -2500pGL3 to -3500pGL3. Also of interest, sequence analysis has identified several putative elements within the promoter including AP-1, AP-2, and SP-1 elements, as well as binding sites for Pitx1, Pitx3, Sp1, and SF-1 (Figure 4).

Materials and Methods

Promoter Sequencing:
• Inverse PCR was utilized to generate additional 5’ flanking sequence of the porcine GnRHR gene. Quasi promoter 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:
• murine gonadotrope-derived cell line, T3-1, was maintained in DMEM with 5% FBS, 5% horse serum, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, and 2 mM glutamine at 37°C in a humidified 5% CO2 in air environment.
• Two million cells were plated in individual wells of a 6-well plate the afternoon prior to transfection.
• 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:
• In preliminary studies, luciferase reporter 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 full-length promoter (-5000) was significantly lower than that of positive controls (murine -600pGL3), leading to future studies with additional 5’ flanking sequence.

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 T3-1 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 T3-1 cell line were performed to compare constructs containing either 1800 bp of the porcine or 600 bp of the murine GnRHR promoter fused to the cDNA encoding luciferase (-1800pGL3 and murine-600pGL3). The murine-600pGL3 vector was well-established as a positive control in T3-1 cells.
• The robust luciferase levels achieved in cells transfected with the -5000pGL3 indicated that this in vivo model 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 T3-1 cell line (Figure 3).
• Large decreases in luciferase activity between deletion constructs indicate that important elements are likely to be located in the following regions: -5000pGL3 to -1000pGL3, -1000pGL3 to -500pGL3, and -2500pGL3 to -3500pGL3.
• Also of interest, sequence analysis has identified several putative elements within the promoter including AP-1, AP-2, and SP-1 elements, as well as binding sites for Pitx1, Pitx3, Sp1, and SF-1 (Figure 4).

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.