DIFFERENTIAL REGULATION OF THE PORCINE GONADOTROPIN-RELEASING HORMONE RECEPTOR (GnRHR) GENE BETWEEN LINES OF SWINE DIVERGENT FOR OVULATION RATE IS CONFERRED BY ELEMENTS WITHIN 1400 bp OF 5' FLANKING REGION

E.A. McDonald, R.A. Cederberg, and B.R. White

Department of Animal Science, University of Nebraska-Lincoln

Abstract

The interaction of gonadotropin-releasing hormone (GnRH) with its receptor on the surface of gonadotrope cells within the anterior pituitary gland represents a key point for regulation of reproductive function. In the pig, the GnRHR gene is located on chromosome 8, in close proximity to a quantitative trait locus for ovulation rate. Thus, the GnRHR gene is both a positional and physiological candidate for genes influencing ovulation rate. We have isolated 5100 bp of 5' flanking sequence for the porcine GnRHR gene from two lines of swine with increased ovulation rates, Chinese Meishan (M) and a line selected on an index of ovulation rate and embryo nic survival (I), as well as a control (C) white crossbred line. As previously reported, transient transfection assays in gonadotrope-derived aT3-1 cells with vectors for each line containing 5100 bp of 5' flanking region fused to the cDNA encoding luciferase resulted in a 2 to 4-fold increase in luciferase activity (I and M, respectively) over the C line ($P < .05$). Vectors containing sequential deletions of the full-length promoters for each line were constructed and transfected to identify a breed specific element(s). Differences in luciferase activity between lines were maintained following reduction of the proximal promoters from 5100 to 1900 bp. Sequence analysis of the -1900 promoters from each line revealed numerous putative elements and base changes between M and C groups. To examine the importance of this region in differential expression of the GnRHR gene, the 500 bp between -1900 and -1400 bp of C promoter were replaced with the same sequence from the M promoter, and vice versa. Transient transfections indicated no difference between promoter "swap" constructs and their respective constructs containing native M and C sequence. Further 500 bp deletions of the -1900 promoters from each line indicated that differential activity was lost when promoters were reduced to 1000 bp of 5' flanking region. Sequence analysis of the -1400 to -1000 bp region upstream of the translational start site revealed multiple elements that may play a role in transcriptional regulation of the GnRHR gene between pig lines, including NF-Y, GATA-1, Sp1, and C/EBP. Thus, further analysis remains to determine the importance of these elements in differential regulation of the GnRHR gene in lines of swine divergent for ovulation rate.

Introduction

GnRH is produced in the hypothalamus of the brain to act on gonadotrope cells located in the anterior pituitary gland, and results in the production and secretion of the gonadotropin hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Binding of GnRH to its receptor on the surface of gonadotrope cells causes the up-regulation of at least four genes. These include the a-subunit, common to LH and FSH, as well as a unique ß-subunit for each hormone. Additionally, binding of GnRH to its receptor stimulates the up-regulation of the gene for its own receptor. Consequently, GnRH and GnRHR represent a key interaction for regulation of reproduction. Our laboratory is interested in transcriptional regulation of the porcine GnRHR gene and its possible effects on ovulation rate. Towards this end, we have chosen to use lines of swine divergent for ovulation rate. Studies were thus conducted using: 1) the Chinese M breed which has approximately 4-5 more piglets per litter, largely due to ovulation rate; 2) the I line, ovulating 7 more oocytes than control animals; and 3) the C line, an unselected white crossbred line of pigs. We have isolated approximately 5100 bp of the 5' flanking region of the porcine GnRHR gene for all three lines of swine. Transient transfections into a gonadotrope-derived cell line using vectors containing the full-length sequence for all three lines of swine indicated a significant increase in luciferase (Luc) values for both lines with increased ovulation rates, M and I, over that of C. Reduction of the proximal promoter from 5100 to 1900 bp relative to the translational start site maintained a 2-4 fold increase (I and M, respectively) over that of C. Sequence analysis of the promoter revealed multiple regions of discrepancy between the three lines, especially between 1900 and 1400 bp of 5' flanking sequence. Within this region, a 22 bp deletion was detected in the M 5' flanking region that was absent in both the C and I lines. The present study aims to isolate the specific regions responsible for the difference in promoter activity among the three lines of swine.

Methods

- Inverse PCR was used to identify additional 5' flanking sequence from that reported by Jiang et al, 2001.
- Specific primers were utilized to isolate the promoter from genomic DNA for each line of swine.
- Vectors were constructed containing the full-length promoter fused to the cDNA encoding luciferase, with deletion constructs prepared from the full-length promoter.
- Transient transfections were performed in αT3-1 cells, a gonadotrope-derived cell line, using a liposome-mediated protocol. Vectors containing a constitutively active promoter (RSV) fused to the gene encoding β-galactosidase were cotransfected as an internal control.
- Following 24 hr of transfection, cells were harvested and luciferase and β-galactosidase activity measured according to manufacturer's instructions.
- Luciferase values were divided by β-galactosidase to adjust for transfection efficiency.
- Results reflect triplicate values from at least three transfections using different plasmid preparations.

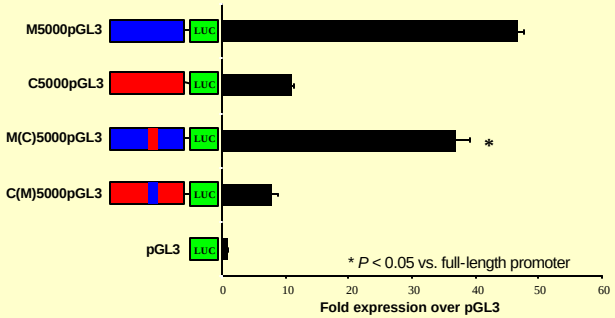

Figure 1. Transient transfection of aT3-1 cells with GnRHR gene promoter "swap" constructs for M and C lines of swine. Transfections were performed using luciferase vectors containing the full-length M and C promoters (M5000pGL3 and C5000pGL3), these vectors with the 500 bp region between -1900 and -1400 "swapped" between lines [M(C)5000pGL3 and C(M)5000pGL3] or promoterless control (pGL3).

Results

To determine the significance of the 22 bp deletion in the M 5' flanking sequence, a "swap" experiment was performed between M and C promoters. The 500 bp located between -1900 and -1400 was replaced with the analogous region from the promoter for the other line. Results indicated that although there was a significant decrease in luciferase activity for the M(C)5000 promoter compared to the full-length M promoter, the difference remained dramatically higher than the C5000 promoter (Figure 1). In addition, luciferase activity of cells transfected with the C(M)5000pGL3 construct did not differ from cells containing the C5000 vector. These results suggest that the element(s) conferring differential regulation of the GnRHR gene promoter among lines of swine is not located in the -1900 to -1400 region of proximal promoter. Further deletion studies were performed, creating vectors containing 1900, 1400, 1000 and 500 bp of 5' flanking region for each line. Results revealed significant differences in luciferase activity between constructs containing 1900 or 1400 bp of proximal promoter for M, I and C lines (Figure 2). However, reduction of the promoter to 1000 bp eliminated differences in luciferase activity, suggesting the presence of an element involved in differential regulation of the GnRHR gene promoter between pig lines.

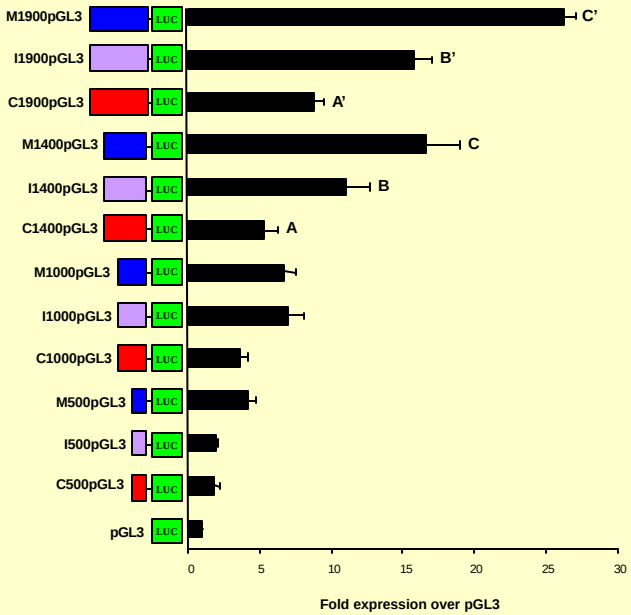

Figure 2. Transient transfection of aT3-1 cells with vectors containing progressively less 5' flanking sequence for the GnRHR gene promoter from M, I and C lines of swine. Transfections were performed with luciferase vectors containing either 1900, 1400, 1000 or 500 bp of promoter from each line of swine and promoterless control (pGL3). Bars for each deletion construct with unique letters are different from one another ($P < 0.05$).

Summary

Deletion studies with the 5100 bp 5' flanking region of the porcine GnRHR gene from three lines of swine divergent for ovulation rate (M, I, and C), as well as "swap" experiments between those lines with the largest differences in promoter activity (M and C), suggested that an element(s) responsible for differential regulation of the GnRHR gene was located between 1400 and 1000 bp upstream of the translational start site. Sequence analysis of this region revealed putative *cis*-acting elements, including binding sites for C/EBP and Sp1. Further deletion studies and mutational analysis of this region are required to elucidate element(s) responsible for differential regulation of the GnRHR gene promoter in lines of swine with divergent ovulation rates. Additionally, electrophoretic mobility shift assays will be performed to identify transcription factors that bind to these elements.