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Abstract

The interaction between GnRH and its receptor is critical to regulation of reproductive function. Additionally, the porcine GnRH receptor (GnRHR) gene is located near a quantitative trait locus for ovulation rate. Thus, the GnRHR gene represents both a physiological and positional candidate for genes influencing ovulation rate. Our laboratory has constructed luciferase reporter vectors containing 5118 bp of the GnRHR gene promoter from 3 lines of swine with divergent ovulation rates; Control, Index and Meishan. Transient transfection of these constructs into gonadotrope-derived alphaT3-1 cells revealed significant differences in luciferase activity among promoters from the three pig lines. The Meishan proximal promoter contains two single-bp alterations at -1234 and -844 bp compared to the Control/Index promoters. The alteration at -1234 bp forms a unique binding site for the p65/p52 subunits of NF-kappaB and an Sp1-like protein whereas the alteration at -844 bp binds GATA-4. Transient transfection of vectors containing block replacement mutations of these binding sites reduced luciferase activity by 20% compared to the full length Meishan promoter. Additional 5' deletion studies identified an enhancer region located at -1810/-1666 bp of proximal promoter. Electrophoretic mobility shift assays (EMSA) utilizing radiolabeled oligonucleotide probes spanning this region indicated specific binding complexes for oligonucleotides comprising the -1779/-1749 and -1700/-1679 bp regions of the Meishan and Control/Index promoters. Sequence analysis of the region between 1749 and 1779 bp upstream of the translational start site revealed a putative binding site for SF-1. Addition of an antibody directed against SF-1 abrogated binding of the complex. Within the region between -1700/-1679 bp of 5' flanking sequence, the Meishan promoter contains a single-bp substitution compared to the Control/Index promoter. Analysis of this region identified potential NF-kappaB and GATA elements in the Meishan and Control/Index promoters. An unlabeled oligonucleotide with consensus GATA binding sites competed the DNA:protein complex for the Meishan probe whereas the addition of an unlabeled NF-kappaB consensus oligonucleotide abrogated binding of the complex for the Control/Index probe. Next, we added antibodies directed against the p50, p52, and p65 subunits of NF-kappaB as well as GATA-1, -2, and -4. Antibodies specific for the p52 and p65 subunits of NF-kappaB resulted in a supershift in the Control/Index oligonucleotide whereas GATA-4 antibody resulted in a supershift of the protein complex bound to the Meishan oligonucleotide. To examine the functional significance of these elements, we constructed vectors containing a block replacement mutation of the GATA-4 binding site within the context of the full length Meishan promoter (μ GATA-4pGL3) and the NF-kappaB binding site within the context of the full length Control promoter. Transient transfections were performed in the alphaT3-1 cell line with μ GAT-A4pGL3, μ NF-kappaBpGL3 and vectors containing the corresponding full length promoters from each line, as well as promoterless control. The GATA-4 block replacement mutation reduced luciferase activity by 49% compared to the full length Meishan promoter whereas the NF-kappaB block replacement mutation reduced luciferase activity by 60% compared to the full length Control promoter. Thus, a complex upstream enhancer located at -1810/-1666 bp of proximal promoter, containing both cell- and line-specific elements, contributes to differential activity of the GnRHR gene promoter in swine lines divergent for ovulation rate.

Introduction

We have isolated 5118 bp of 5' flanking region for the porcine GnRHR gene from three lines of swine with different ovulation rates. Transient transfection of a gonadotrope-derived cell line using vectors containing the full-length promoter for all three lines of swine indicated a significant increase in luciferase values for both lines with increased ovulation rates, Meishan and Index, over that of Control. Reduction of the proximal promoter from 5118 to 1900 bp relative to the translational start site maintained a 2-4 fold increase in luciferase activity (Index and Meishan, respectively) over the Control promoter. Further, an enhancer located at -1810/-1666 bp of 5' flanking region was identified by additional 5' deletion studies. The present study aimed to isolate specific regions responsible for differences in promoter activity among the three lines of swine within this upstream enhancer located at -1810/-1666 bp of proximal promoter.

Materials and Methods

- Vectors were constructed containing the full-length promoter of the porcine GnRHR gene from three lines of swine with different ovulation rates fused to the cDNA encoding luciferase, with block replacement constructs prepared in the context of the full-length promoter.
- Transient transfections were performed in gonadotrope-derived alphaT3-1 cells using a liposome-mediated protocol (Fugene6, Roche Molecular). The reporter vector (0.9 μ g) was cotransfected with a control vector, RSV beta-galactosidase (0.1 μ g).
- Three transfections were completed in triplicate using DNA from three different plasmid preparations.
- Luciferase (Promega) and beta-galactosidase (Tropix) assays were performed.
- Luciferase values were divided by beta-galactosidase activity to normalize for transfection efficiency and means were expressed as fold changes over promoterless control.
- Means for adjusted luciferase activity were compared using least significant differences.
- EMSAs were performed using 32P-labeled oligonucleotides spanning the upstream enhancer region located at -1810/-1666 bp of proximal promoter and crude nuclear extracts obtained from alphaT3-1 cells.

Meishan/Control/Index: **TGAGTTTGGTTGTTTTCACAGGCATTAG**

Figure 1. Sequence spanning the -1779/-1749 bp region of the porcine GnRHR gene promoter. Highlighted box represents the SF-1 binding site.

Control/Index: **AACCCCATATTCCTACTGAGA**

Meishan: **AACCCCATATCTAGGCACTAA**

Figure 2. Sequences spanning the -1700/-1679 bp region of the Control/Index and Meishan GnRHR gene promoters. Highlighted box in the Control/Index represents the NF-kappaB binding site and in the Meishan represents the GATA element.

Results

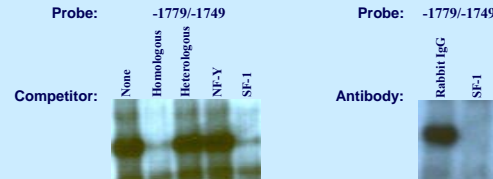


Figure 3. Oligonucleotides spanning the region located at -1779/-1749 bp of the porcine GnRHR promoter binds SF-1 in alphaT3-1 cells. EMSA analysis with alphaT3-1 nuclear extracts revealed that the specific complex binding to the -1779/-1749 oligonucleotide was ablated by the addition of an unlabeled consensus SF-1 oligonucleotide. Addition of an antibody directed against SF-1 abrogated the specific binding complex.

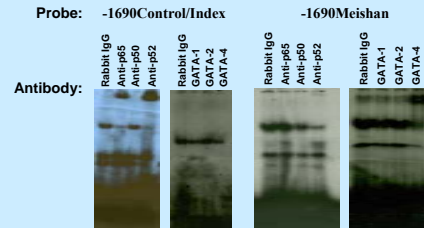


Figure 4. Oligonucleotides spanning the -1690 bp alteration in the Control/Index and Meishan GnRHR promoters bind the p65/p52 subunits of NF-kappaB and GATA-4, respectively. EMSA was performed with nuclear extracts from alphaT3-1 cells and oligonucleotides spanning the -1690 bp substitution between the Control/Index and Meishan swine lines. Addition of antibodies directed against p65 and p52 subunits of NF-kappaB resulted in a supershift of the specific complex binding to the Control/Index oligonucleotide. However, the binding complex associated with the Meishan oligonucleotide was supershifted by an antibody specific for GATA-4.

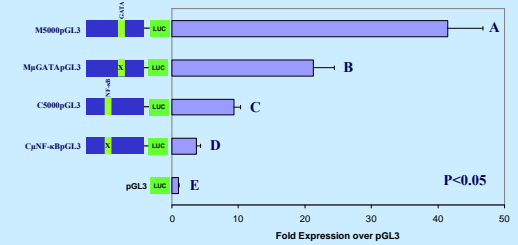


Figure 5. Functional significance of the NF-kappaB and GATA-4 binding sites specific to the Control and Meishan promoter, respectively. Transfections were performed with luciferase (LUC) vectors containing the native full-length Control and Meishan promoter, a block replacement mutation of the NF-kappaB and GATA-4 binding site within the context of the Control and Meishan full-length promoter, respectively, or promoterless control (pGL3). Differences are indicated by bars with unique letters ($P < 0.05$).

Conclusions

A T to C bp substitution at -1690 bp in the Meishan GnRHR promoter forms a GATA element, allowing the GATA-4 transcription factor to bind at this location (Figure 6). However, the Control/Index promoter has a T residue at this location, resulting in binding of the p65/p52 subunits of NF-kappaB. These unique elements in the GnRHR promoters of Control/Index and Meishan swine lines help explain differential promoter activity within alphaT3-1 cells. The SF-1 binding site within the Control and Meishan promoters located at -1779/-1749 bp of proximal promoter allows binding of the transcription factor, SF-1. Thus, the SF-1 binding site may contribute to the cell-specific promoter activity within alphaT3-1 cells.

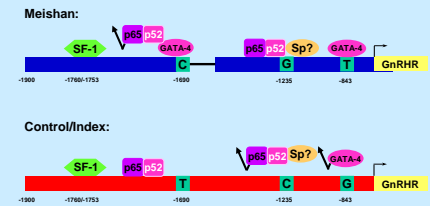


Figure 6. Working model for the line specific activity of the porcine GnRH receptor gene promoter.

Acknowledgments

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