The Search for the Bovine Relaxin-Like Factor Receptor in the Non-Pregnant Cow

Brittany Bell, Christine Bross, Alan Eldred, Michael J Fields

ABSTRACT

Relaxin has long been known as a pregnancy hormone that works on the connective tissues of the reproductive tract and has proven vital to the parturition process by causing the softening of the cervix and relaxation of the pubic ligaments. It also is suspected of having a role in the implantation period in early pregnancy. In order to better understand this sensitive period of pregnancy, previous research has focused on identifying the bovine version of the hormone. To date, no such factor has been found. However, when injected with porcine relaxin, pregnant cows show a classical response to the hormone. It was hypothesized that cattle must possess a relaxin-like factor instead of the relaxin hormone, and that the factor must act through its own receptor (LGR8). In addition, the relaxin-like factor receptor must be closely analogous to the receptor for relaxin (LGR7) to produce a relaxin-induced response. The present study focused on locating the relaxin-like factor receptor in bovine reproductive tissues. Primers were developed through the Primer 3 online generator using a computer-predicted sequence for LGR8. RNA was extracted from bov taurus corpora lutea (CL) and follicles, and bos taurus uterine endometrium and myometrium. RT-PCR was performed and the resulting cDNA was fluoresced on a 1.5% agarose gel. The resulting cDNA from the CL consistently produced a band of 400 – 550bp. It was subsequently cloned and is currently awaiting sequencing. The cDNA from the follicular tissue also had recurrent results. These findings reveal possible expression of LGR8 in the bovine CL and follicle and support the important role it plays in the early period of implantation and pregnancy.

INTRODUCTION

Relaxin as a pregnancy hormone

Relaxin has long been known as a pregnancy hormone because of it many reported functions during gestation. Studies conducted as early as 1950 revealed relaxin’s ability to sustain myometrial quiescence in estrogen-primed guinea pigs1. Probably more notable is relaxin’s role in elongating the interpubic ligament before parturition by acting on the collagen fibers and causing dissolution and tissue remodeling2. Other past studies have concluded that relaxin, acting of the same pathway, plays an imperative role in cervical softening before parturition3 and assists in placental expulsion after parturition by remodeling the tissues of the placentomes during late pregnancy4. Many other non-reproductive roles have been discovered for the hormone,
but the current study focuses on the magnitude of its functions in the implantation and parturition events of pregnancy.

Most sensitive of these two events is that of implantation, which is described as a time of high embryonic loss in both beef and dairy cattle. In fact, the economic losses associated with embryonic mortality in the peri-implantation period have become a significant concern in the cattle industry. Researchers have postulated uterine-conceptus asynchrony and failure of maternal recognition of pregnancy as likely factors attributing to embryonic loss. Pinpointing the factors involved in the sequence of events preceding the implantation process of pregnancy would enable scientists to correlate the high rates of early embryonic loss with certain abnormalities, thus providing an effective method for reducing the occurrence of this economic issue within the cattle industry.

Relaxin in the implantation period

Some of the earliest markers of implantation in mammalian species are insulin growth factors (IGFs) and insulin growth factor binding proteins (IGFBPs), both of which are related to relaxin in the same family of proteins. These IGFs and IGFBPs are secreted from the endometrium and act on pre-implantation bovine embryos in vitro. In primates, specific relaxin receptors have been localized in the endometrium during the peri-implantation period, leading to the hypothesis that relaxin could function as a signal for implantation before attachment takes place. In other embryo attachment studies, rabbits demonstrated a conceptus-mediated implantation process that limits attachment to areas where direct embryo-endometrial cell-cell contact occurs. Interestingly, endometrial relaxin was detected and found to be restricted to these implantation sites, leading again to the possible paracrine effect of relaxin at the site of embryo attachment. Furthermore, research has shown that chorionic gonadotropin (CG) causes decidualization of the endometrial stromal cells in primates, where the endometrial stroma transforms itself into a dense cellular matrix. The effect of this hormone is increased by the presence of relaxin.

In addition to embryo implantation, vascularization of the embryo after attachment is another important event in early pregnancy. Embryonic infiltration of the maternal vasculature is imperative for conceptus survival. In this step of the implantation process, relaxin has been found to induce the expression of vasculature endothelial growth factor (VEGF) in endometrial cells in vitro and generate changes of maternal vasculature in vivo. Thus, relaxin demonstrates an important potential role in the three major events of embryo implantation: endometrial recognition of the embryo, decidualization of the endometrial stromal cells, and embryonic vascularization.

The search for the bovine strain of relaxin

A bovine-specific strain of relaxin has not been identified to date, yet, previous research has demonstrated that cows injected into the cervical os with porcine relaxin produce a classical response: increasing the rate of pelvic area expansion and dilating the cervical sphincter. Researchers have suggested three possibilities for why
the bovine sequence has not been identified: a) ruminants may express quantities of relaxin that are too low to be detectable by today’s technology, b) the ruminant strain may be too evolutionarily distant to be identified by probes based on other species, or c) a relaxin gene may simply not be present in most ruminants18. In fact, sheep possess a relaxin-like pseudogene, characterized by an mRNA transcript that codes for stop codons in every possible reading frame. Translating this mRNA to protein would produce a greatly truncated, non-functional peptide19. The only known ruminant to express relaxin is the camel20. Phylogenetically, sheep are shown to have evolved after the camel, but before cattle5, suggesting the presence of a pseudogene or the complete absence of a bovine strain of relaxin.

Relaxin as it relates to cryptorchidism

Relaxin-like factor (RLF), also known as insulin-like factor 3 (INSL3)21, came to rise after being discovered in the Leydig cells of the porcine testis. It is a member of the insulin family of peptides and shares many similarities to other members of the same family22. To date, RLF cDNA clones have been detected and sequenced in the testes and other tissues of several mammalian species, including humans23, bovines24, sheep25, rats26, dogs27, deer28, and horses29. Linked to the physiological aspect of testicular descent, RLF causes cryptorchidism in the male when absent, where the gubernacular ligament fails to shorten30. Furthermore, when an RLF-expressing construct was introduced into female mice, the mice developed an ovary that was forced into the inguinal region by a developed gubernaculum, demonstrating the role of RLF in helping the testes to pass into the scrotum31. Deletion of the receptor for RLF, determined to be leucine-rich repeat-containing G protein-coupled receptor 8 (LGR8), reversed the phenotype caused by the RLFoverexpression, indicating that the LGR8 is the only receptor for RLF32.

However, due to its unusually high luteal expression in the ruminant when compared with other species during the peri-implantation process, Bovine RLF is now being evaluated for its ability to function as a compensatory protein in the absence of a relaxin gene. Previous experiments proved groundbreaking in being the first to localize RLF within the bovine corpus luteum (CL)33. The study at hand utilized polymerase chain reaction (PCR) techniques to search for the RLF receptor, LGR8, in the tissues of the cow.

MATERIALS AND METHODS

Primer design

An automated computational analysis predicted the bos taurus sequence for LGR8, which was downloaded from the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) at listing number XM_583603.1. The template was then transcribed to the Primer3 Online Primer generator version 0.3.0 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for the designing of the mRNA probes. Primer analysis was performed by the freeware program Oligo Analyzer 1.1.2, downloaded at www.uku.fi/~kuulasma/OligoSoftware, and the primers with the largest base pair predicted products and the least self-
annealing qualities possible were selected for probing. These were the Fields – 8.1 forward (catctgcatctggatgtgg) and reverse (cccaggaggtgatgtgtct) primers as shown in Table 1. In addition to these initial primers, the Bathgate – 8 forward (ctgccatcatcatctgtgtgtc) and reverse (ttggaagcaagggtatcttc) primers were designed by Dr. Ross Bathgate of Howard Florey Institute at the University of Melbourne, Victoria, Australia, and included in the analysis.

Later, an updated version of the predicted bos taurus sequence was downloaded from the NCBI website at listing number XM_583603.2. This second sequence was reevaluated and once again transcribed to the Primer3 Online Primer Generator. After analysis in the Oligo Analyzer, an additional set of primers was chosen. These were the BB – 1 forward (ggtgtgacattgctgcttt) and reverse (ttctggtctcagctctcctct) sequences and the BB – 2 forward (ccgtgtctcattcctctc) and reverse (ctgtgttgtggaggttacccc) sequences.

Porcine LGR8 forward (gttctcgatgccatctgctg) and reverse (cgctggttgtgcagcagctg) primers [33] were borrowed from Dr. Frank Bartol at the Department of Animal Sciences, Cellular and Molecular Biosciences Program at Auburn University, Auburn, Alabama, and used with porcine reproductive tissues to compare and serve as a test for laboratory technique.

**RNA extraction**

Reproductive tissues were harvested post mortem from the reproductive tract of non-pregnant cows and non-pregnant gilts at various stages of cyclicity. Bovine and porcine uterine samples were divided by manual separation into myometrium and endometrium, and bovine ovarian samples were separated into follicular and luteal (CL) tissues. The tissues were then frozen with liquid nitrogen to –80°C until RNA extraction was necessary. RNA extraction was then performed using the RNAqueous-Midi Large Scale Phenol-Free Total RNA Isolation Kit by Ambion.

The tissues were removed from –80°C and homogenized in 10 mL of Lysis/Binding solution with a Fisher Scientific PowerGen 700 Homogenizer, kindly provided by Dr. Lokenga Badinga’s research facilities in the Department of Animal Sciences, University of Florida, at approximately 25,000 RPM for 5 minutes each. Following homogenization, the lysates of each tissue sample were pelleted by centrifugation for 8 minutes at 8,000 RPM, removing any cellular debris. The lysates were subsequently diluted with an equal amount of a 64% ethanol solution. This dilution was then passed through a glass filter provided in the kit at a rate of 3 to 5 drops per second. Following the filtering, air was then passed through to clear the filter of any foamy debris. A wash of the filter containing the extracted RNA was using a wash solution at a rate of 3 to 5 drops per second, followed by a second and third wash, using only 70% of the first volume of wash solution. Next, the RNA was eluted from the filter using a heated elution solution provided in the kit. A total of 1.5 mL of the hot elution solution was passed through the filter in thirds and collected in a clean vial.

**RT-PCR**
Following extraction, the template RNA was prepared for real-time polymerase chain reaction (RT-PCR) amplification using the Platinum Quantitative RT-PCR ThermoScript One-Step System by Invitrogen. The template RNA was combined with the ThermoScript Reaction mix, the ThermoScript Plus Enzyme mix, and the forward and reverse primers as named and listed in Table 1.

### Table 1.
Primer names and sequences used in the study.

<table>
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<tr>
<th>Name</th>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>NCBI Record</th>
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<td>CATCTGCATCTGGATTGTGG</td>
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<td>XM_583603.1</td>
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<tr>
<td>Bathgate - 8</td>
<td>Bovine LGR8</td>
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<td>TGGAAAGCAAGGTATTCTC</td>
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<tr>
<td>Bartol - 8</td>
<td>Porcine LGR8</td>
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<td>CTTGTGTAGTGGAGGACC</td>
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</table>

Amplification was completed in an Eppendorf MasterCycler, kindly provided by the research facilities of Dr. Alan Ealy at the Department of Animal Sciences, University of Florida. cDNA synthesis and pre-denaturation were completed in one cycle at 60°C for 30 minutes followed by 5 minutes at 95°C. Forty cycles of PCR amplification were subsequently carried out following the Conventional RT-PCR Protocol set forth in the Invitrogen ThermoScript One-Step System Manual, each with denaturing, annealing, and extending periods of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 5 minutes, respectively.

The results were then analyzed by electrophoresis on a 1.5% agarose gel. A TrackIt 100 BP ladder (Invitrogen) was included in the gel, and the gel was electrified for approximately 45 minutes. After analysis with a specialized UV light, photographs were taken of the resultant gels with a Polaroid digital camera from the facilities of Dr. Alan Ealy of the Department of Animal Sciences, University of Florida, and then cropped and magnified with Adobe PhotoShop.

**Cloning**

The Invitrogen Zero Blunt TOPO PCR Cloning Kit was used to prep the cDNA for sequencing. Other tissues had failed to produce adequately sized cDNA products. Therefore, only cDNA from the CL was prepared for sequencing. The cDNA template was inserted into the TOPO vector and then incorporated into Chemically Competent *E. coli*. The solution was then spread on pre-warmed, previously prepared LB-agar plates and incubated overnight at 37°C. One colony was selected from each plate and then transferred to test tubes for the miniprep step.
Minipreps were taken of each incubated cell-containing solution following the protocol described in the Quantum Prep Plasmid Miniprep Kit by Bio-Rad Laboratories. The Plasmid-containing cells were pelleted, resuspended, and lysed, and then pelleted again to remove cell debris. Next, the plasmid DNA was suspended in the Quantum Prep matrix and washed twice. Finally, the plasmid DNA was eluted from the filter and subsequently stored at −20°C. After performing an analysis by gel electrophoresis, as shown in Figure 2, the plasmid DNA samples were sent to the University of Florida Cancer and Genetics Institute for sequencing.

RESULTS

The RT-PCR reactions consistently produced products, but the product sizes varied greatly throughout the study. Table 2 shows a summary of the following results. As illustrated in Figure 1, in the first gel, where the porcine tissues were included as a reference, a band of approximately 400 base pairs (bp) was visualized in the lane for porcine endometrium. This was ~200bp more than the product size of 198bp previously reported [34]. However, the lane with the bovine CL sample using the Fields–8.1 primer visualized a band of ~550bp, which is very close to the expected value of 530bp, as predicted by the Primer3 generator when using the Fields–8.1 primer. No product was visualized for the lanes containing porcine myometrium, bovine endometrium with the Bathgate LGR8 primer, bovine endometrium with the Fields–8.1 primer, or in the lane containing bovine CL with the Bathgate LGR8 primer.

Next, a second gel was run with the same primer combination for the bovine CL. Once again, a product was visualized, but it now showed a band size of ~400bp. And, like the previous gel, the bovine endometrium showed no result with the Fields–8.1 primer.

The third gel was able to reproduce the ~400bp band in the bovine CL lane, but a smear was present. However, the bovine endometrium lane now produced a faint smear at about 300bp.

In an effort to clarify the seemingly decreasing product band of the bovine CL tissue, different primers were used in the fourth gel. Primers BB–1 and BB–2, as described in Table 1, were each combined with a sample of bovine CL and bovine endometrium. None of these tests resulted in product bands on the gel.

In the fifth gel, we returned to using the Fields–8.1 primer, but also included some lanes with the BB–1 and BB–2 primers. The bovine CL with the Fields–8.1 primer did not visualize any results, but the same tissue did show a fuzzy band less than 100bp when combined with the BB–1 primer and a band of ~100bp when combined with the BB–2 primer. Here, the first tests for the follicular tissue were also observed. Bovine follicular tissue was combined with the Fields–8.1 primer and produced a band of approximately 300bp.

The sixth gel showed more promising bovine follicular tissue results. Samples combined with the Fields–8.1
primer visualized a band of ~350bp. Combined with the BB – 1 primer, the sample showed a band of ~200bp with a smear down the gel, compared to the expected range of 543bp. Here also, the bovine CL produced results once again. Samples combined with the Fields – 8.1 primer expressed a band in the 100 to 200bp range. And the CL sample combined with the BB – 2 primer produced a band at ~300bp, with a smear down past 100bp. This band is in comparison to the predicted size of 456bp for that primer.

In the final gel, we used freshly manufactured primers to ensure a proper product in the PCR reaction. This seventh gel exhibits the results produced by these fresh primers, indicated by a (B) behind the name. Lanes 5, 7, and 11 all contained the Fields – 8.1 (B) primer, and each showed a strong band at ~600bp that smeared down the gel to 100bp.

Lanes 5 and 7 were samples of endometrium and CL, respectively, but were from a different cow (cow 38) than all the previous samples (cow 8159) in all the previous gels. Lane 11 was CL from the same cow as all the previous samples. Lanes 6, 8, and 10 were each of samples combined with the BB – 1 (B) primer. These three lanes each showed a fuzzy band at the bottom of the gel, similar to that of a primer-dimerization, where the primers bind to themselves instead of the selected sequence. The tissues tested in these lanes were CL from cow 38 and endometrium and CL from cow 8159, respectively.

Figure 1. Results of the first four electrophoresis gels showing cDNA products. (A) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = Porcine myometrium, Lane 4 = Porcine endometrium, Lane 5 = Bovine Endometrium, Lane 6 = Bovine CL. (B) Lane 1 = ladder, Lane 2 = empty, Lane 3 = positive
control, Lane 4 = negative control, Lanes 5, 6 and 9 = tests for outside research, Lane 7 = Bovine CL, Lane 8 = Bovine endometrium. (C) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lanes 4 and 5 = tests for outside research, Lane 6 = Bovine CL, Lane 7 = Bovine endometrium. (D) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lanes 4, 5, 6 and 7 = tests for outside research, Lane 8 = Bovine CL with BB – 1 primer, Lane 9 = Bovine CL with BB – 2 primer, Lane 10 = Bovine endometrium with BB – 1 primer, Lane 11 = Bovine endometrium with BB – 2 primer.

Figure 2. Results of gels 5, 6, and 7 (A, B, and C) and the DNA gel (D) showing the expected size of the sequence cloned after enzyme digestion. (A) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lane 4 = 18S, Lane 5 = PCR Optimum Control, Lane 6 = Bovine CL with Fields – 8.1 primer, Lane 7 = Bovine CL with BB – 1 primer, Lane 8 = Bovine CL with BB – 2 primer, Lanes 9, 10, and 11 = tests for outside research, Lane 12 = Bovine follicle with Fields – 8.1 primer. (B) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lanes 4 = Bovine follicle with Fields – 8.1 primer, Lanes 5, 6, 8, and 11 = tests for outside research, Lane 7 = Bovine follicle with BB – 1 primer, Lane 9 = Bovine CL with Fields – 8.1 primer, Lane 10 = Bovine CL with BB – 2 primer. (C) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lanes 4 and 5 = tests for outside research, Lane 6 = Bovine CL, Lane 7 = Bovine endometrium. (D) Lane 1 = ladder, Lane 2 = positive control, Lane 3 = negative control, Lanes 4, 5, 6 and 7 = tests for outside research, Lane 8 = Bovine CL with BB – 1 primer, Lane 9 = Bovine CL with BB – 2 primer, Lane 10 = Bovine
endometrium with BB – 1 primer, Lane 11 = Bovine endometrium with BB – 2 primer.

Table 2. Gel Results. Chart summarizing the product results of the RT-PCR reactions. Compares visualized results with the expected product ranges.

<table>
<thead>
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<th>Gel</th>
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<th>Results</th>
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<td></td>
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<td>Bov. CL 8159</td>
<td>BB - 1 (B)</td>
<td>&lt;100bp</td>
<td>543 bp</td>
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The endometrium and myometrium samples did not significantly express LGR8 and therefore were not included in
the cloning and sequencing process. The follicular tissue and the CL each proved promising through repeated results. However, later RNA extractions of the follicular tissue did not produce significant yields. Therefore, the follicular tissue was not included in the sequencing either. Results of the sequencing are currently pending.

**DISCUSSION**

Early embryonic loss is fast becoming a large concern in the cattle industry, as it leads to reproductive inefficiency and loss of capital. In fact, recent studies have shown that nearly 30-40% of total pregnancies are lost within the first 20 days of gestation [35]. Asynchrony between uterine and embryonic maturation has been posed as a primary cause for early bovine embryonic loss. In an effort to better understand the pathways to embryonic implantation, scientists are searching for the link between the hormones present during the peri-implantation period and the location of the hormone receptors. Uncovering these receptors would aid in the sequencing of events pertinent to embryo implantation and maternal recognition of the conceptus, identifying the normal environment required by the conceptus, and labeling abnormal characteristics carrying a high risk for loss. Information of this nature would enable industry leaders to minimize their risk of embryonic loss through selection for better genetic predisposition.

The current study has shown that there is a high possibility for the RLF receptor, LGR8, to be present in the bovine CL. This data coincides with the studies that found RLF in the ovary [32, 37]. Speculation would suggest that where the protein hormone is released, the receptor for paracrine action would be nearby. Also, understanding the tissue remodeling the ovary undergoes at ovulation to transform the follicle into a corpus luteum for the maintenance of pregnancy supports the idea that a relaxin-like molecule would play an important role. However, confirmation of this data is still needed in the form of a sequence that is homologous to the computer-generated sequence as listed on NCBI. BLAST, an online sequence evaluation program, will be used to compare the sequenced product from the PCR reaction to other known sequences already posted in the NCBI database. The evaluation program will give percent similarities for sequences that are homologous.

Further research is necessitated by the promising results the bovine follicular tissue also produced. The RNA extraction method should be repeated on fresh follicular tissue until proper concentrations can be obtained and results can be sequenced. Also, the search for better primers should be ongoing. Different combinations of primers may work better for the different reproductive tissues selected. The same methods of research could be repeated with different primer sequences. In addition, more variables should be added to pinpoint the exact time during the cycle of during pregnancy the receptor is being expressed. Tissues harvested from different times in the estrus cycle and throughout pregnancy should be evaluated.

In closing, we have demonstrated the possible presence of LGR8 in the bovine CL. These findings may be indicative of the role relaxin-like factor plays in the implantation process of pregnancy.
ACKNOWLEDGEMENTS

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REFERENCES


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