CHARACTERIZATION OF PROGESTERONE RECEPTOR (PGR) MRNA AND PROTEIN ISOFORMS IN THE ENDOMETRIUM OF CYCLIC AND PREGNANT PIGS

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CHARACTERIZATION OF PROGESTERONE RECEPTOR (PGR) MRNA AND PROTEIN ISOFORMS IN THE ENDOMETRIUM OF CYCLIC AND PREGNANT PIGS

presented by Erin Marie Sellner

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
°F	Degrees Fahrenheit
3β-HSD	3 beta – hydroxysteroid dehydrogenase
AF1	Activation function domain – 1
AF2	Activation function domain – 2
AF3	Activation function domain – 3
AI	Artificial insemination
AR	Androgen receptor
BLAST	Basic local alignment search tool
Bp	Base pair
BrdU	Bromodeoxyuridine
C terminus	Carboxy terminus
cAMP	Cyclic adenosine monophosphate
CARM 1	Coactivator-associated arginine methyltransferase 1
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CL	Corpora lutea
cm	Centimeter
COC	Cumulus – oocyte complex
C t	Cycle threshold
cRNA	Complementary ribonucleic acid

CUEDC2	CUE domain containing 2
CYP11a	Cytochrome P450
d	Day
DAB	Diaminobezidine
DBD	Deoxyribonucleic acid binding domain
DIUI	Deep intrauterine insemination
DMSO	Dimethylsulfoxide
DNAse 1	Deoxyribonuclease 1
E2	Estradiol
E6-AP	E6 – Associated protein
ECL	Enhanced chemiluminescence
EPD	Expected progeny difference
ER	Estrogen receptor
ERα	Estrogen receptor, alpha isoform
ERαKO	Estrogen receptor alpha knock-out
ERβ	Estrogen receptor, beta isoform
ERE	Estrogen response element
EST	Expressed sequence tag
FSH	Follicle stimulating hormone
GE	Glandular epithelium
GnRH	Gonadatropin releasing hormone
GnRHR1	Gonadatropin releasing hormone receptor 1
GR	Glucocorticoid receptor

HAT	Histone acetyltransferase
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
IGFBP – 1	Insulin – like growth factor binding protein – 1
IGF – 1	Insulin – like growth factor 1
IHC	Immunohistochemistry
IL-1β	Interleukin - 1β
IUI	Intrauterine insemination
kb	Kilobase
kDa	Kilodalton
LB	Luria-Bertani broth
LBD	Ligand binding domain
LDS	Loading sample buffer
LE	Luminal epithelium
LH	Luteinizing hormone
LIMS	Laboratory information management system
LSM	Least squares mean
Lys	Lysine
M	Molar
µg	Microgram
µl	Microliter
mg	Milligram

ml	Milliliter
mm	Millimeter
mM	Millimolar
MMP2	Matrix metalloproteinase 2
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleic acid
MUC1	Mucin – 1
N	Number of replicates
N terminus	Amine terminus
NAI	No artificial insemination
NBA	Number born alive
NCBI	National Center for Biotechnology Information
NF-κB	Nuclear factor – kappa B
Ng	Nanogram
NLS	Nuclear Localization Signal
ORF	Open reading frame
OXTR	Oxytocin receptor
P4	Progesterone
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F_{2alpha}
PGR	Progesterone receptor

PGR-A	Progesterone receptor, A isoform
PGR-A/B	Progesterone receptor, A and B isoform common region
PGR-B	Progesterone receptor, B isoform
PGR-C	Progesterone receptor, C isoform
PKI	Protein kinase 1
Pmol	Picomole
Poly(A)	Polyadenylated
PRAKO	Progesterone receptor, isoform A knock-out
PRBKO	Progesterone receptor, isoform B knock-out
PRE	Progesterone response element
PRKO	Progesterone receptor knock-out
PR-M	Progesterone receptor, M isoform
PRMT 1	Protein arginine methyltransferase 1
Proc GLM	General linear model procedure
PR-S	Progesterone receptor, S isoform
PR-T	Progesterone receptor, T isoform
PSI	Proteasome inhibitor
PVDF	Polyvinylidene fluoride
qRT-PCR	Semi-quantitative real-time polymerase chain reaction
RelA(p65)	Nuclear factor – kappa B subunit
RLM – RACE	RNA ligase mediated – rapid amplification of cDNA ends
SAS	Statistical analysis software
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SNP	Single nucleotide polymorphism
Sp1	Stimulatory protein 1
SRC	Sarcoma proto-oncogene tyrosine kinase
SSA	*Sus scrof*a autosome
SSC	Saline sodium citrate
StAR	Steroidogenic acute regulatory protein
SUMO	Small ubiquitin-like modifier
SWI/SNF	Switch/Sucrose non fermentable
TBS-T	Tris buffered saline with Tween
TRAP/DRIP	Conjugal transfer protein/*drosophila* integral protein
TRBP/ASRC II	TAR RNA binding protein
Tyr	Tyrosine
UTJ	Uterotubal junction
UTR	Untranslated region
V	Volts
W/V	Weight per volume
X-gal	X-galactosidase
YWHAG	Tyrosine – 3 – monooxygenase/tryptophan 5 monooxygenase

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ACADEMIC ABSTRACT

Disappearance of the progesterone receptor (PGR) from the uterine luminal epithelium (LE) is essential for cyclicity and pregnancy in pigs. In humans, three PGR mRNA isoforms (PGR-A, PGR-B and PGR-C) arise from alternative transcription start sites, conferring distinct biological functions. The objective was to identify and characterize PGR mRNA and protein isoforms in the pig during the estrous cycle and pregnancy. Primer pairs for porcine PGR were developed from porcine genomic and human mRNA sequences and used to amplify porcine PGR fragments from cDNA. The porcine PGR mRNA sequence generated is 4.3 kb with 84% identity to human, 79% identity to murine and 86% identity to bovine PGR mRNA. The transcription initiation site for the full-length PGR transcript was determined to be located between 1 and 470 nucleotides by PCR amplification of cDNA. Three in-frame open reading frames were identified and putative protein sequence inferred. The porcine PGR protein is 934 amino acids in length with 84% overall identity to human PGR protein. The porcine PGR protein was 75% identical to human PGR protein within the A/B (variable) domain and was 97% identical to human PGR protein within the C, D, and E domains. Homology in mouse and cow was also higher within the C, D, and E domain over the variable domain.

Polymorphic sites within the sample population and between generated mRNA and genomic sequences were identified and resultant effects on protein sequence inferred.

Primer pairs for quantitative real-time PCR (qRT-PCR) were originally developed from porcine genomic and mRNA sequence and used to amplify porcine PGR fragments from cDNA. Based on cDNA sequencing and porcine genomic sequence, the porcine PGR mRNA is 4.3 kb. RT-PCR primers for PGR-B (5'-TCAGACTGAAGTCGGGGAAC-3' and 5'-GGGTGAAATCTCCACCTCCT-3') and PGR-AB (region common to both PGR-A and PGR-B; 5'-GCTCCATGGTTCCACTTCTG-3' and 5'-GATGGGCACGTGGATAAAAT-3') were developed to study PGR regulation in endometrial tissue from cyclic (days 0, 5, 7.5, 10, 12, 13, 15, 17) and pregnant (days 10, 12, 13, 15, 17) pigs (n = 53 samples; minimum of 4 pigs per status per day). There was a tendency for an effect of d on uterine PGR-B expression ($P < .10$), because PGR-B mRNA fold change was greater on day 0 (d 0; 0.52 ± 0.07) and d 5 (0.51 ± 0.07) compared with d 7.5 (0.31 ± 0.07) and d 15 (0.30 ± 0.05) (remaining d were intermediate). The PGR-AB mRNA remained low through d 13 (0.13 ± 0.01; d 0 to 13; cyclic and pregnant) and increased on d 15 in both pregnant (0.90 ± 0.07) and cyclic (0.41 ± 0.07) pigs ($P < 0.001$). The PGR-AB mRNA remained elevated in pregnant pigs on d 17 (0.33 ± 0.06). The existence of PGR-B and possibly PGR-A isoforms were detected by Northern Blot analysis. PGR-C, however, was not detected. Transcription initiation sites were detected in PGR by RPA: *PGR-B.1*, 291-314; *PGR-B.2*, 379; *PGR-A*, 1046; *PGR-C.1*, 2559; *PGR-C.2*, 2631. We conclude that PGR isoform mRNA abundance may change during the estrous cycle and pregnancy potentially leading to functional differences in PGR action. We also observed the

presence of PGR-B, PGR-A and PGR-C in qRT-PCR and ribonuclease protection assay analyses, although some transcripts may be too unstable to be consistently detected by Northern blot analysis.

Three PGR antibodies were identified that were expected to cross-react with porcine PGR: a PGR-B-specific antibody and two PGR-AB antibodies which targeted the region of PGR protein common to PGR-B, PGR-A and PGR-C isoforms. The presence of PGR-B and PGR-A proteins was confirmed by immunoblotting, but none of the antibodies were able to detect PGR-C. Additionally, the molecular weights of the proteins identified, though consistent with previous findings in humans and pigs, were observed to be greater than predicted from amino acid sequence. Finally, abundance of PGR-B and PGR-A isoforms was assessed on d 0, d 8 and d 12 (n = 3 samples per day), with no differences detected between the days. The three antibodies were then used for immunohistochemistry localization of PGR isoforms to specific cell types. The PGR-B specific antibody detected strong nuclear staining in the LE and uterine glandular epithelium (GE), decreasing in intensity from d 8 to d 12. Both PGR-AB antibodies, however, detected no nuclear staining in the LE and only sporadic staining of the stroma nuclei. Antibodies to PGR-AB also showed cytoplasmic staining in the uterine LE, GE and stroma that was not observed using the PGR-B antibody, with strongest staining on the apical surface of the LE on d 12. The PGR-A isoform, therefore, may have a more diffuse cytoplasmic staining than the strictly nuclear staining of PGR-B. Taken together, these results may indicate varying levels of biological activity rather than protein abundance of the two isoforms on days 8 and 12 of the estrous cycle in porcine endometrium.

The project concludes that porcine PGR has high nucleotide and amino acid conservation with human, mouse and cow sequences. The project concludes that abundance of mRNA and abundance of protein are, at times, uncoupled in this system, with PGR-C present as an mRNA transcript but not a translated protein. Abundance of mRNA and abundance of protein, at other times, appear to act in tandem, with PGR-B mRNA and PGR-B protein nuclear staining in the LE and GE both decreasing from d 8 – d 12. New questions to be investigated were identified for further study to increase understanding of PGR isoform expression in the porcine endometrium.

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CHAPTER I

PROJECT INTRODUCTION

The uterus must undergo dynamic changes during both the establishment of pregnancy and during the estrous cycle, both in proliferation and differentiation of cells into deciduas (Larsen et al., 2002). In both uterine states, high, sustained concentrations of progesterone (P4) stimulate the rapid removal of progesterone receptor (PGR) from the uterine luminal epithelium (LE; Tseng and Zhu, 1997; Harduf et al., 2009; Kurita et al., 2000a, 2001; Mote et al., 1999; Arnett-Mansfeld et al., 2004; Spencer and Bazer, 2002). This ligand induced down-regulation of PGR is an essential event for return to cyclicity in non-pregnant gilts and sows and permits attachment of the conceptuses in pregnant animals. Failure to achieve down-regulation of PGR could have severe repercussions, particularly in maintenance of pregnancy. Previous studies have demonstrated the disappearance of PGR from the LE and glandular epithelium (GE) in both the estrous cycle and in early pregnancy in other species, and the research presented here was based on experimental preliminary demonstration of this expression pattern specifically in pigs (Geisert et al., 2006; Ross et al., 2010).

In a study designed to examine whether repression by the RelA(p65) subunit of nuclear factor κB (NF-κB) was responsible for PGR down-regulation in the porcine uterus, i*n situ* hybridization with a PGR-specific cRNA probe was used to investigate

cellular localization of PGR transcript expression at distinct time points during the estrous cycle and early pregnancy (Ross et al., 2010). Tissues samples examined were collected from hysterectomized gilts on d 0 (estrus), d 5, d 7.5, d 10, d 12, d 15 and d 17 of the estrous cycle and on d 10, d 12, d 15, and d 17 of early pregnancy. The results were concordant with known PGR expression in other species, demonstrating the presence of PGR transcripts from days 0 to 5 in the LE, GE and stroma. Rapid loss of transcript abundance in both pregnant and cyclic gilts between days 7.5 and 12 were coincident with rising serum P4 concentrations produced by corpora lutea. Expression of PGR transcript expression was completely depleted on day 13 in the LE and GE. The LE and GE expression of PGR recovered from undetectable amounts beginning d 15 and continuing on d 17 in cyclic gilts, concurrent with regression of the corpora lutea. In pregnant gilts, this recovery did not occur on d 15 or d 17 but remained low. The PGR mRNA expression in the stroma remained constant among treatments (Ross et al., 2010).

These results demonstrate the dynamic changes in PGR mRNA expression in the porcine endometrium with implications for fertility and maintenance of pregnancy in pigs. The experiment does not differentiate, however, between variant PGR isoforms, which may be regulated independently, have different signaling pathways and confer distinct biological functions. The study also does not investigate PGR expression at the level of protein or protein functionality, both of which may not be concordant with changes in PGR mRNA transcription. Further study is required to elucidate the patterns of expression specific to each isoform toward the purpose of more comprehensive understanding of the role of PGR in cyclicity and pregnancy.

A 2007 USDA NRI grant by R.D. Geisert and M.C. Lucy based on the above results proposed further investigation of the interaction of NF-κB with PGR down-regulation in the porcine endometrium. The project was funded with support from National Research Initiative Grant no. 2007-3520-17836 from the USDA Cooperative State Research, Education, and Extension Service. A portion of this project focused on the central hypothesis that PGR mRNA and protein isoforms exhibit different patterns of expression throughout the phases of the estrous cycle and during early pregnancy in the porcine endometrium. The first specific aim of the project was to generate the mRNA and amino acid sequences of porcine PGR, perform *in silica* analysis of gene and protein conservation between similar species and identify sequence characteristics. The second specific aim was to develop a method for the detection of porcine PGR mRNA isoforms in the pig uterus and characterize the expression of these isoforms at different time points in during the estrous cycle and in early pregnancy. The third specific aim of the project was to confirm the presence of PGR isoform protein variants and then to detect and characterize PGR isoform expression at the protein level at different stages of the estrous cycle, both in total endometrium and in individual cell types.

CHAPTER II

LITERATURE REVIEW

INTRODUCTION

Although almost 95% of ovulated oocytes in swine are fertilized, only 50 – 70% of these fertilizations result in viable offspring (Pope, 1994). In swine, early embryonic death is responsible for much of this discrepancy between number of oocytes fertilized and number of live births per litter. In an experiment conducted by Geisert et al. (2007), by day 30 of gestation, sows that consistently produced small litters had 5.1 fewer viable embryos than their counterparts of average fecundity. Additionally, between days 9 and 18 of gestation, the rate of embryonic mortality in average producing sows was 17% (Anderson, 1978). Taken together, these results demonstrate the role of early embryonic mortality as a major contributor to suboptimal litter size.

Indeed, Geisert and Schmitt (2001) proposed that U.S. sows have the genetic potential to produce up to 14 pigs per litter, while the National Agriculture Studies Service reports that in small (< 99 head) swine operations only 7.5 pigs are produced per litter, with large (> 5,000 head) operations achieving only 9.3 pigs per litter (http://www.nass.usda.gov/). There is, therefore, the potential for a substantial economic improvement, in increasing early embryonic survival and thus litter size in the swine

industry. Fewer sows would be required to meet current consumer demands for pork products, requiring less monetary input toward the maintenance of fully-grown sows in feed and housing costs. Increased litter size would also allow producers to obtain an increased number of progeny from gilts and sows with the best genetic potential, which may increase the rate of genetic progress yielded per generation in the commercial swine industry. Finally, environmental regulations on animal solid waste disposal and gaseous emissions are becoming more stringent, so maintaining fewer gilts and sows which are the primary generators of waste products on a gestation and farrowing operation would decrease the amount of governmentally regulated waste to be handled by producers. This would result in more environmentally-friendly production operations, as well as lower monetary penalties assessed to operations unable to meet waste disposal regulations. Investigation of uterine status during this early period of pregnancy may yield better understanding of how the uterus may contribute to early embryonic mortality, and thus elucidate a potential for improvement in the reproductive rate of swine (Geisert and Schmitt, 2001).

CHARACTERIZATION OF PGR, P4 AND E2 IN UTERINE DYNAMICS

Estradiol (E2) is a steroid hormone produced by the granulosa cells of a growing follicular cohort during the follicular phase of the porcine estrous cycle (d 13 – 14 to d21). Estradiol has a positive feedback mechanism on hypothalamic production of gonadotropin releasing hormone (GnRH). Secreted GnRH then stimulates follicle

stimulating hormone (FSH) release from the anterior pituitary, which continues to stimulate the growth and maturation of the follicular cohort, producing greater concentrations of E2 as they grow to ovulatory size. Production of estradiol decreases rapidly prior to ovulation as granulosa cells become luteinized.

Estradiol also has a positive effect on the expression of PGR. As E2 increases from d 13 – 14 through d21, there is a subsequent increase in PGR in the uterus, particularly the LE of the endometrium (Diaz and Wiltbank, 2004; Guthrie, 2005). Thus, PGR is highly expressed prior to ovulation and P4 begins to be secreted by luteinized follicular cells. At the level of the uterus, E2 stimulates the proliferation of endometrial cells. Finally, the E2 surge created by preovulatory follicles results in the expression of estrus behavior, such as standing heat, in pigs (Senger, 2003).

During the luteal phase of the porcine estrous cycle beginning on d 0, high concentrations of E2 produced by large preovulatory follicles induce a surge of luteinizing hormone (LH) that stimulates the rupture of the follicles, inducing ovulation. Each ruptured follicle transitions to a corpus hemorrhagicum and finally a corpus luteum. Follicular theca cells become LH-responsive small luteal cells and follicular granulosa cells become $PGF_2\alpha$-responsive large luteal cells. Both cell populations are steroidogenic and secrete P4 during the luteal phase of the porcine estrous cycle.

Progesterone is a steroid hormone which acts at the level of the uterus to maintain quiescence. Progesterone exerts its effect through specific binding to the ligand binding domain (LBD) of its receptor, PGR (Conneeley et al., 2002). Ligand binding stimulates a conformational change in PGR and dissociation of the receptor from a variety of complexed proteins, including heat shock proteins (Leonhardt and Edwards, 2002). The

shedding of this protein complex allows the nuclear localization signal (NLS) to direct PGR into the nucleus of the cell. Once in the nucleus, PGR isoforms can then form either homo- or heterodimers and bind to a 15 bp recognition site (PRE: progesterone response element) in the promoter region of progesterone-responsive genes. Once bound, PGR attracts coactivators to affect the rate of transcription (Conneeley et al., 2003; Lee et al., 2006).

High, sustained concentrations of P4 induce the rapid removal of PGR by d 10 in both cyclic and pregnant pigs (Geisert et al, 2006). The steroidogenic acute regulatory protein (StAR) enzyme is activated by LH, P4 and PGE2 to stimulate the production of P4 from cholesterol within the luteal cells (Rekawiecki et al., 2005). Within the mitochondria, cholesterol is cleaved by cytochrome p450 side chain cleavage enzyme (CPY11a) to pregnenolone which is exported from the mitochondria and subsequently cleaved by 3β-hydroxysteroid dehydrogenase (3β-HSD) to P4 which is secreted from the luteal cells. Secreted P4 works at the level of the hypothalamus to decrease the pulsitility of GnRH through a negative feedback loop (Senger, 2003) and also at the level of the uterus to antagonize oxytocin and thereby maintain uterine quiescence (Spencer et al., 2006).

EARLY PREGNANCY IN THE SOW

Fertilization occurs at the oviductal ampullary-isthmus junctions in the porcine bicornuate uterus (Senger, 2003). Conceptuses then migrate throughout the uterine horns

and establish non-overlapping spacing (Dzuik, 1985). The porcine conceptuses undergo rapid development and hypertrophy between d 10 – 12 post conception (Anderson, 1978). Spherical embryos (10 mm) elongate to a tubular shape approximately 14 mm in length. Within a few hours the embryos elongate from the tubular stage to a filamentous conformation, measuring more than 150 mm in length before apposition and attachment to the uterus (Geisert et al., 1982a). Pigs have a non-invasive, epitheliochorial placenta that is fully established by 18 – 20 days after conception (Blair et al., 1991; Senger, 2003).

In the pregnant porcine uterus, the developing conceptuses secrete proteins and steroid hormones that act on the uterus to prepare the endometrial LE for conceptus attachment (Franczak and Bogacki, 2009). Porcine conceptuses secrete a short, transient pulse of E2 in the lumen of the uterus on d 11-12 after estrus followed by a prolonged E2 secretion after d 15 (Geisert et al., 1982a). In the absence of conceptuses, $PGF_{2\alpha}$ produced by the uterus drains into the uterine veins and is circulated systemically to cause regression of the CL (Bazer and Thatcher, 1977). Thus, during the normal estrous cycle in the absence of a pregnancy, the uterus secretes $PGF_{2\alpha}$ in an endocrine manner. Conceptus release of E2 stimulates the uterus to redirect the secretion of $PGF_{2\alpha}$ from the endometrium to the uterine lumen, thus protecting the CL on the ovaries from the luteolytic activity of $PGF_{2\alpha.}$ This biphasic mode of secretion from systemic in non-pregnant pigs to luminal sequestration of the lipid hormone in response to the signal for maternal recognition of pregnancy is known as the endocrine – exocrine theory (Bazer and Thatcher, 1977). Another prostaglandin, E2, (PGE2) plays a role in maintenance of the CL as an anti-luteolytic/ luteotrophic signal. In response to sustained high P4 in

conjunction with the E2 signals from the conceptus, PGE2 secretion from the uterine luminal epithelium and conceptuses is increased in the uterus from days 11 – 14 (Geisert et al., 1982a), increasing the serum PGE2: $PGF_{2\alpha}$ ratio and further protecting the CL from luteolysis (Davis and Blair, 1993; Ford and Christenson, 1991).

Synchronization of uterine stage with stage of conceptus development is essential for successful implantation of porcine embryos (Spies et al., 1959; Day et al., 1963; Pope, 1988). Both pulses of E2 are necessary and sufficient for maternal recognition of pregnancy. Asynchrony of E2 expression with the uterine environment results in failure of the conceptuses to survive to the stage of apposition and attachment to the uterus (Ross et al., 2007). The timing of these pulses must be synchronized with the status of the uterus to facilitate normal implantation (Polge, 1982). Aberrant expression of E2 resulting in asynchrony with the uterine environment results in total early embryonic loss, as Geisert et al. (2006) demonstrated by administering exogenous E2 prematurely on days 9 and 10 rather than normal conceptus expression on days 11 and 12.

When the pulses of E2 from the developing conceptuses are secreted, expression of PGR protein has already been down-regulated in the LE. This may be an essential prerequisite for E2 to be effective in signaling maternal recognition of pregnancy (Geisert et al., 1994). Indeed in rhesus monkeys, sheep, and mice (Okulicz and Scarrell, 1998; Spencer and Bazer, 1995; Tan et al., 1999, respectively) PGR is down-regulated in the LE to confer uterine receptivity prior to implantation of the conceptus, as well. This suggests that this pattern of expression is not coincidental but crucial. What is not known, however, is whether total PGR or merely a specific isoform down-regulation from the LE is necessary and sufficient for E2 signaling from the conceptus for the

maternal recognition of pregnancy.

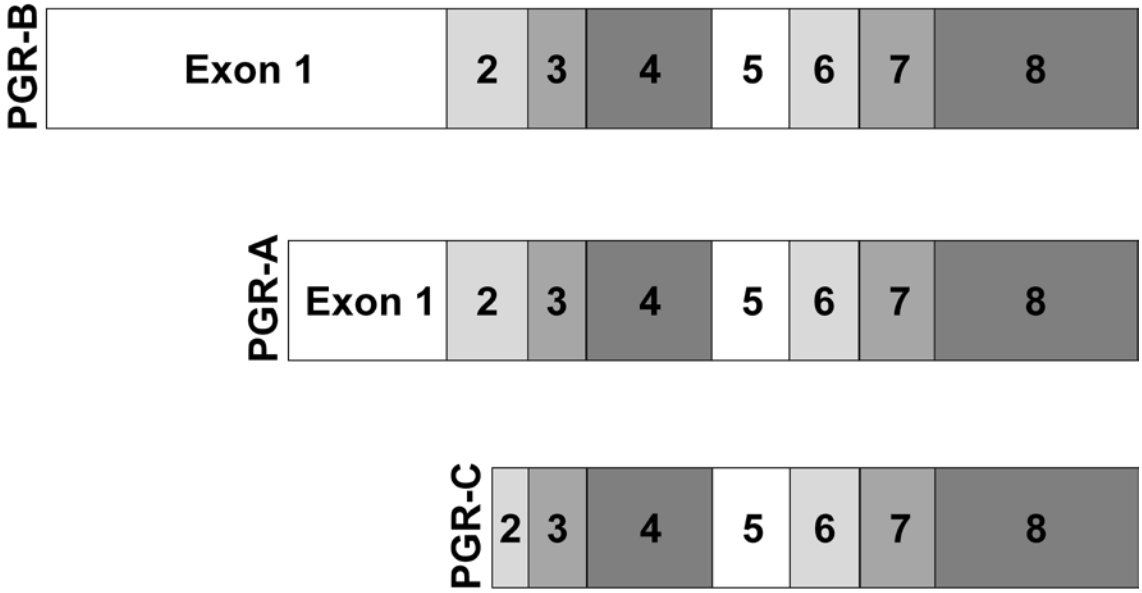
GENE STRUCTURE AND MRNA

In swine, the PGR gene is located on SSA 9p13-11 (Hu et al., 1998). The gene is nearly 100 kb in length and contains eight exons. In humans, mouse and rat, the PGR gene is located on chromosomes 11, 9 and 9, respectively (National Center for Biotechnology Information). In each of these species the PGR gene contains 8 exons and 7 introns. The PGR-B isoform transcript is encoded by all eight exons and arises from a transcription initiation site located at the 5' end of exon 1. The PGR-A isoform transcript arises from a downstream transcription initiation site located in exon 1 and has its own separate promoter. Although neither promoter sequence contains a classical estrogen response element, transcription of both isoforms is inducible by estrogen (Kastner et al., 1990). The PGR-C isoform arises from a third promoter with a transcription initiation site putatively located at the 3'-most end of exon 1 (Figure 2.1). In fact, even PGR-C has been demonstrated to be inducible by estrogen (Kraus and Katzenellenbogen, 1993).

A classical mechanism for the regulation of PGR suggests that transcription of PGR is up-regulated by the presence of estrogen receptor 1 (ESR1 or estrogen receptor alpha (ERα)). Conversely, expression of PGR has an inhibitory effect on ER1 expression, thereby indirectly inhibiting expression of the oxytocin receptor (OXTR; Spencer et al, 2006). After periods of high P4 concentration, P4 itself down-regulates expression of the PGR through a currently unknown mechanism.

There may be evidence, however, for differential regulation of different PGR transcripts. Binding of coactivators to the PGR-A promoter region for instance, acts as a

Figure 2.1 Representations of the protein coding regions of porcine PGR-B, PGR-A and PGR-C isoform mRNA transcripts. Exons 1 through 8 are denoted. The transcripts encode in-frame translation start sites, resulting in identical C-terminal protein products with N-terminal truncations of the protein from PGR-B isoform to PGR-A isoform to PGR-C isoform.



repressor of the transcriptional activity of the PGR-B isoform (Giangrande et al., 1997; Vegato et al., 1993). Transgenes containing human PGR-A promoter ligated to the chicken PGR-A gene (a potent activator of PGR-B transcription activity *in vivo)* were transfected into chimeric cell cultures. These cell cultures demonstrated abrogation of the chicken PGR-A-induced activation of PGR-B transcription, demonstrating that activation of the classical PGR-A promoter region acts as a repressor of PGR-B isoform transcription (Giangrande et al., 1997).

The presence of PGR-C isoform mRNA and protein remains controversial in the scientific literature. The PGR-C isoform has been characterized in human breast cancer cell lines T47D and HeLa (Wei et al., 1996). The PGR-C isoform has also been characterized as the primary PGR protein isoform expressed at term in the human placenta and was up-regulated in human myometrium during labor, suggesting a potential role in the onset of parturition (Taylor et al., 2005; Condon et al., 2006). In various cell cultures derived from human myometrium and the human breast carcinoma cell line T47D, however, the PGR-C isoform as well as rare transcript variants (PR-M, PR-S, and PR-T) were demonstrated to be artifacts of lowly efficient antibodies, rather than naturally occurring isoform transcripts *in vivo* (Samalecos and Gellersen, 2008).

Alternative splicing between the exons results in several additional variants of PGR protein. Currently five variants have been identified in humans: exon 4-deleted, exon 6-deleted, exon 5,6-deleted, exon 4,5,6-deleted and exon 4,6-deleted transcripts. While the four former transcripts have all been identified, along with full-length mRNA, in the endometrium during all phases of the menstrual cycle the latter transcript is

observed only in clinical cases of women with ovarian endometriosis (Misao et al., 1998; Marshburn et al., 2005).

Regulation of PGR at the level of RNA transcription has been investigated in several E2-responsive tissues, including the granulosa cells of the ovary in the rabbit, an ER positive human breast cancer immortalized cell line (MCF-7) and human endometrial cells (Savouret et al., 1998; Petz and Nardulli, 2000; Clemens et al., 1998, respectively). In a study of human endometrial cells that did not differentiate between isoforms, PGR transcription was induced by binding of ER-β to an estrogen response element (ERE) of the proximal promoter region (Clemens et al., 1998). The presence of estrogen alone, however, was not sufficient to induce increased PGR expression in granulosa cells. The gonadotropins LH and FSH are present at high concentrations during follicular development. These hormones induce the intracellular second messenger cAMP which, acting synergistically with ER, induces transcription of PGR in granulosa cells (Clemens et al., 1998).

In a study using an MCF-7 human breast cancer cell line, a half-ERE/Sp1 binding site in the promoter region of PGR-A was identified *in vivo* using DNAse footprinting (Petz and Nardulli, 2000). By transfecting the promoter elements into a chloramphenicol acetyltransferase reporter vector, Petz and Nardulli (2000) demonstrated that purified Sp1 protein bound to two Sp1 sites in the promoter region of PGR-A which was enhanced by ER binding.

The rabbit is unique to the other mammalian species currently characterized, because they do not use alternative PGR promoters to express alternative isoforms, but

rather separate genes for PGR-B and PGR-A (NCBI). However, an ERE within the primary PGR transcript and correlating to the PGR-A promoter site in other species was identified (Savouret et al, 1998). Binding of ER or an exogenous E2 agonist to this site induced PGR transcription. Using domain deletions in the ER, it was demonstrated that AF2 and not the AF1 domain of ER is necessary and sufficient for binding to the ERE of PGR and inducing PGR transcription. Down-regulation of PGR by P4 was discovered to be mediated by PGR binding via this same ERE in rabbit (Savouret et al., 1998).

The above studies have investigated the activation of PGR transcription, but another method of controlling transcript expression is gene silencing by methylation. In women with endometriosis, it is common for PGR-B expression in the uterus to be down-regulated (Wu et al., 2006). Using methylation-specific microarrays verified by bisulphite sequencing, the promoter region of the PGR-B isoform was confirmed to be hypermethylated in uterine samples from human endometriosis versus control samples. Abundance of the PGR-B transcript was also decreased in these samples, suggesting that in the disease state, the PGR-B DNA promoter is hypermethylated in the human endometrium, decreasing transcription of PGR-B isoform mRNA (Wu et al., 2006). The study identifies mechanisms for both up- and down-regulation of PGR-B at the level of transcription. Binding of E2 to a PGR-A-specific ERE may recruit a unique complement of coactivators as a possible mechanism for inhibiting PGR-B transcription through specific hypermethylation of the PGR-B promoter. In this model, promoter activation of a specific PGR isoform could repress transcription of alternative promoters, thereby regulating transcription of the isoforms independently of one another rather than global up- or down-regulation of all PGR isoforms concurrently.

MRNA EXPRESSION

PGR has been assessed at the mRNA level in a variety of tissues and species including the uterus, oviduct, follicle, vagina, hypothalamus, pituitary, and carcinomas of human, mouse, rat and pig for both total PGR and for isoform-specific expression. Human, mouse and rat uterus have been used to model PGR expression, particularly in the E2-responsive endometrium (Tseng and Zhu, 1997; Harduf et al., 2009; Kurita et al., 2000ab, 2001; Kraus and Katzenellenbogen, 1993; Fang et al., 2002; Sahlin et al., 2006). In cell cultures from hysterectomized human endometrium, ribonuclease protection assays (RPA) detected mRNA for PGR-B and PGR-A to be decreased in GE in response to either P4 or a synthetic progestogen (MPA), while both compounds increased expression of both PGR-B and PGR-A isoforms in the stroma (Tseng and Zhu, 1997). In human endometrium during a normal menstrual cycle, PGR was detected using a PGR-A/B common region probe in the GE and stroma at all phases of the cycle (Harduf et al., 2009). Constitutive stromal expression did not respond to high serum E2 concentration, but had a tendency (not statistically significant) toward decreased expression when E2 concentrations were low. During this period of low serum E2 concentrations in the early proliferative phase of the menstrual cycle, GE expression of PGR decreased significantly from that of the mid-proliferative expression. In human endometrial cell lines known to be either lowly receptive to implantation (HEC-1A) or simulating the highly receptive endometrium during the window of implantation (RL95-2), semi-quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine relative expression of PGR-B isoform mRNA. The ratio of PGR-B expression to GAPDH expression in HEC-1A

cells was 0.084 ± 0.08, while expression in RL95-2 cells was significantly greater (0.44 ± 0.13; Harduf et al., 2009).

Northern blot analyses of mouse uterine LE detected the presence of 4 distinct PGR transcripts at 11.5, 8.7, 6.9, and 4.4 kb in length (Kurita et al., 2000a). In two experiments, the 6.9 kb isoform was most abundant. This transcript was identified as PGR-A, followed in abundance of transcript by the 8.7 kb isoform, identified as PGR-B. Other transcripts were not included in the analysis (Kurita et al., 2000ab, 2001). Expression of both isoforms was high in ERα knockout (ERAKO) mice treated with either oil or E2 (Kurita et al., 2000a). The PGR-B and PGR-A isoforms were similarly expressed in the LE of wild-type mice, but undetectable in E2-treated uterus of wild-type mice (Kurita et al., 2000a). Ovariectomized mice treated with either E2 or E2 plus P4 showed undetectable expression of PGR-B and PGR-A in the LE. These results demonstrate the species specific regulation of PGR by E2, as classical endocrinology theory of the E2/P4 hormone feedback loop suggests that E2 is a potent up-regulator of PGR expression. In mice, however, it has the opposite effect on transcript abundance. In the stroma of intact, ovariectomized, and ovariectomized with E2 supplementation ERKO mice, E2 supplementation was sufficient to induce increased expression of both PGR-B and PGR-A (Kurita et al., 2001). Supplementation of E2 to ERKO mice was also sufficient to induce increased expression of both PGR-B and PGR-A transcript isoforms in vaginal epithelium (Kurita et al., 2000a). This suggests that E2 is able to act through ERβ or another unknown mechanism specifically in the stroma and vaginal epithelium and not in the uterine epithelium.

In rat whole uterus, total PGR abundance was assessed at the mRNA level in

response to E2 stimulation. Using Northern blots to assay abundance, Kraus and Katzenellenbogen (1993) demonstrated a time- and dose- dependent transient up-regulation of PGR in response to E2 stimulation. The mRNA abundance reached its peak with a 6-fold increase in expression 24 hours after E2 stimulation. Treatment with P4 was sufficient to block E2-mediated up-regulation in mRNA (Kraus and Katzenellenbogen, 1993). In the gestating uterus, RPAs were able to demonstrate that PGR-B does not appear to change in abundance throughout gestation, however, total PGR (an amalgam of both PGR-B and PGR-A) significantly increased 24 to 36 hours before parturition (Fang et al., 2002). Using qRT-PCR with probes specific to PGR-B or common to PGR-A/B in total uterine tissues, neither PGR-B nor PGR-A were significantly increased in expression by treatment with P4 (Sahlin et al., 2006). It is important to note that these results are from whole uterine tissue, and cell-specific changes may be undetectable due to dilution of cell type specific variation in total cell milieu.

Human and rat neural tissues have also been assessed for PGR expression. Six mRNA species were detectable in 33 human meningioma samples; 11.4, 6.1, 5.2, 4.5, 3.2, and 2.5 kb (Carroll et al., 1993). Only the 11.4 and 6.1 transcripts seem to correlate to the transcripts present in normal mouse vaginal epithelium, uterine epithelium and uterine stroma, perhaps suggesting that carcinomic state alters transcript size and milieu (Kurita et al., 2000ab, 2001). Using qRT-PCR in rat hypothalamus with probes specific to PGR-B and PGR-A/B, PGR-A/B was constitutively expressed during the estrous cycle, whereas PGR-B fluctuated. The PGR-B transcript was least abundant at diestrus, increasing until 1700 hours at proestrus and declining again until 1300 hours at estrus

(Liu and Arbogast, 2009). The PGR-B transcript isoform was the predominant isoform in the pituitary of prepubertal female rats, as detected by RPA. Abundance of PGR-B isoform transcript was increased in response to E2 supplementation and decreased in response to P4 supplementation (Attardi et al., 2007). These results suggest that unlike mice, rats display classical response of PGR to steroid hormones.

PROTEIN CHARACTERIZATION

Progesterone receptor is a member of the steroid nuclear receptor superfamily, binding to its specific steroid hormone, P4, forming homo- or heterodimers with other family members and binding directly to DNA P4 response elements (PREs) to act as transcription factors of P4-responsive genes. The PGR protein like all superfamily members contains several domains: a DNA binding domain (DBD; C), a hinge region (D) containing the nuclear localization signal (NLS), a P4-specific hormone ligand binding domain (LBD; E) and a dimerization domain (DeGroot and Jameson, 2001). The PGR protein undergoes post-translational modifications. There are 11 identified serine residues that are phosphorylation sites in human PGR, 5 of which are specific to PGR-B (UniProt; Wei et al., 1987). Additionally, there are 3 Lys-Gly sites on which small ubiquitin-like modifier (SUMO) cross-links have been identified, one of which is specific to PGR-B protein (UniProt). Upon protein synthesis, PGR becomes hypermethylated in T47D breast carcinoma cells, reaching 4 phosphate groups per protein in the absence of ligand (~50% phosphorylated) and 9 phosphate groups per protein in the presence of P4

(~100% phosphorylated; Beck et al., 1996). Phosphorylation of specific serine residues affects the stability of PGR protein. Progesterone-dependent phosphorylation of the Ser 294 residue was more pronounced in PGR-B than PGR-A and resulted in increased degradation of the protein by the 26S proteasome (Clemm et al., 2000, Lange et al., 2000; Dennis et al., 2001). Modification with SUMO may likewise alter the responsiveness of PGR to P4 and other factors and affect its rate of degradation (Jones et al., 2006, Abdel-Hafiz et al., 2002).

In humans, three isoforms of the PGR mRNA and protein have been described: PGR-B encoding a 116 kDa protein, PGR-A encoding a 94 kDa protein and PGR-C encoding a 60 kDa protein. Isoforms PGR-B and PGR-A are also characterized at 120 kDa and 94 kDa molecular weights in mice and rats. The PGR-A and PGR-C protein isoforms arise from downstream, in-frame AUG methionine codons with variable transcription initiation sites (Wei et al., 1996). The resultant PGR-A and PGR-C proteins, therefore, are identical to PGR-B with N-terminal truncations. The truncation in the PGR-A isoform is 164 amino acids from the N-terminal of PGR-B. The translational start site for PGR-C however, is located within exon 2. Both PGR-B and PGR-A isoform proteins contain the DBD, whereas this domain has been truncated in PGR-C so that the PGR-C isoform cannot act as a transcription factor. Thus, PGR-C may act as a repressor of PGR-B and PGR-A action by competitive binding with P4 and also by dimerizing with and modulating transcription factor activity of both PGR-B and PGR-A without acting as a transcription factor (Wei et al., 1996; Condon et al., 2006).

Both PGR-B and PGR-A contain activation function (AF) domains. The AF-1 is located at the N-terminus of the DBD and is present in PGR-B and PGR-A. The AF-2 is

located C-terminally in the LBD and is present in all three PGR isoforms. A third activation function domain (AF-3) is located N-terminally to PGR-A and is specific to PGR-B. Additionally, an inhibitory domain (ID) is located specifically in the N-terminus of PGR-A only (Li and O'Malley, 2003).

The PGR-C protein may be the dominant isoform of PGR in the myometrium of late gestation in rats (Fang et al., 2002) and during parturition in mice (Condon et al., 2006), as well as in the human placenta (Taylor et al., 2005). The PGR-C may also be localized exclusively to the cytosolic fraction of cells (Li and O'Malley, 2003).

Wen et al. (1994) propose that PGR-B and PGR-A act as transcription factors through different pathways, even when effecting the same response. Parallel mutations in PGR-B and PGR-A affected the efficacy of PGR-B in activating transcription at the MMTV promoter, but did not alter the activity of PGR-A (Wen et al., 1994).

A thermodynamic analysis demonstrated that the PGR-B isoform displays cooperative binding to several PREs to activate transcription. PGR-A binding to several PREs is much more weakly cooperative in comparison. The increased binding affinity of PGR-B may determine the isoform-specific preferential activation of P4-responsive genes with multiple PREs in the promoter region (Connaghan-Jones et al., 2007). An independent study also concluded that PGR-B is more efficient at activating PRE-induced expression in mouse mammary tumor cells than PGR-A (Kastner et al., 1990).

Some known targets of PGR-induced transcription include SRC proteins, HATs, SWI/SNF, TRBP/ASRC II, E6-AP, CARM I, PRMT I, and TRAP/DRIP (Li and O'Malley, 2003). The PGR acts with coactivators to initiated RNA polymerase II to the transcription initiation sites of these genes. The PGR has also been implicated in the

splicing, elongation and termination of target genes through both genomic and non-genomic mechanisms (Li and O'Malley, 2003).

The PGR-B and PGR-A isoforms are actively degraded in the presence of sustained P4 by the proteasome. In a human breast cancer cell line T47D, a protein (CUE domain containing 2; CUEDC2) interacts directly with PGR isoforms to target them for ubiqitination and subsequent degradation by the proteasome (Zhang et al., 2007). Likewise, in several structures of the rat brain, treatment with a proteasome inhibitor (PSI) blocked P4-induced down-regulation of PGR-B and PGR-A by the 26S proteasome (Camacho-Arroyo et al., 2002).

PROTEIN EXPRESSION

The PGR has been assessed at the protein level in a variety of tissues and species including endometrium, myometrium, oviduct, whole uterus, mammary gland and non-reproductive tissues of human, non-human primate, mouse, rat, dog, cow, sea lion and pig for both total PGR and for isoform-specific expression (Kyrou et al., 2009; Tseng and Zhu, 1997; Harduf et al., 2009; Mote et al., 1999; Arnett-Mansfield et al., 2004; Merlino et al., 2007; Jackson et al., 2007; Silvestri et al., 2007; Narkar et al., 2006; Kurita et al., 2000a; Kurita et al., 2001; Shao et al., 2006; Zeng et al., 2008; Aupperlee, 2007; Kraus and Katzenellenbogen, 1993; Ilenchuk and Walters, 1987; Vermeirsch et al., 2002; Berkquist et al., 1981; Jacobs et al., 1980; Colegrove et al., 2009). Human, mouse and rat uterus have been used to model PGR expression, particularly in the estrogen-responsive

endometrium (Kyrou et al., 2009; Tseng and Zhu, 1997; Harduf et al., 2009; Mote et al., 1999; Arnett-Mansfield et al., 2004; Merlino et al., 2007). In a clinical study of women suffering from chronic infertility, cyclicity was simulated via exogenous hormones and endometrial biopsies both on the day of menstruation and on either d3 or d 5 after GnRH treatment following menstruation or at the time of hCG treatment following menstruation (Kyrou et al., 2009). Sections of endometrial biopsies were assessed by IHC with a PGR-A/B antibody. Total PGR-A/B was significantly increased in the GE and stroma of the second biopsy on d 5 versus d3 and hCG treated samples. Total PGR-A/B was also significantly increased in the second biopsy of each treatment group over the first biopsy taken on the day of menstruation. This demonstrates the ability of exogenous hormones to simulate *in vivo* PGR expression in human endometrium (Kyrou et al., 2009).

While PGR protein was lost from LE of human endometrium in response to P4, protein expression of both isoforms PGR-B and PGR-A was actually increased in the human endometrial stroma, as demonstrated by Western blot and immunohistochemistry (Tseng and Zhu, 1997; Harduf et al., 2009; Mote et al., 1999; Arnett-Mansfield et al., 2004). Insulin-like growth factor I (IGF-I) mediated similar effects. Interestingly, cyclohexamide was as effective as progesterone antagonist RU486 in blocking P4-mediated up-regulation of PGR-B and PGR-A protein in the endometrial stroma, implying that this up-regulation by P4 is mediated indirectly via another protein that must be newly synthesized upon binding of P4 to PGR. Immunohistochemistry (IHC) detected PGR in the GE and a scattered pattern of expression in the stromal cells during the proliferative phase of the menstrual cycle. By the midsecretory phase of the menstrual cycle, PGR was very low to undetectable in the glands and more intense in the stroma.

Western blot analysis with PGR-B and PGR-A/B antibodies detected a decrease in PGR-B (116 kDa) and PGR-A (81 kDa) in the GE and an increase in both isoforms in the stroma when treated with a progestogen. Supplementation with E2 increased both isoforms in both stroma and GE (Tseng and Zhu, 1997).

In a Western blot to confirm prior mRNA results, Harduf et al. (2009), detected three PGR isoforms (B at 116 kDa, A at 82 kDa, and C at 60 kDa). All PGR isoforms were detected in the nucleus and were absent from the cytosol. The PGR-B:PGR-A ratio was greater in the lowly receptive HEC1A LE cells than the highly receptive RL95-2 LE cells (Harduf et al, 2009). This is in contrast with an increased presence of PGR-B mRNA expression in RL95-2 cells, indicating both that PGR-A may be necessary for successful implantation and an uncoupling between PGR mRNA abundance and protein abundance.

Another study of PGR expression in human endometrial cells collected by hysterectomy at different phases of the menstrual cycle used a saturating concentration of PGR-B specific antibody in conjunction with a PGR-A/B antibody (Mote et al., 1999). Since immunoblotting with the PGR-B antibody alone did not differ in the intensity of PGR-B detection, PGR-A was detected specifically by subtraction. Expression of both isoforms in the GE was high in the early-late proliferative phase and decreased to a nadir at the late secretory phase. In stroma, PGR-A was most abundant in the mid-proliferative phase and least abundant at the early proliferative and late secretory phases, while PGR-B displayed low, constitutive expression (Mote et al., 1999).

Finally, using IHC and confocal microscopy analysis with dual staining of PGR-B

and PGR-A/B antibodies, more thorough examination of PGR expression within the nucleus of cells was possible (Arnett-Mansfield et al., 2004). Both isoforms were similar in abundance during the proliferative phase, transitioning to predominantly PGR-A expression in the early secretory phase followed by PGR-B dominance in the mid-secretory phase and finally low expression during the late secretory phase. Subnuclear examination determined furthermore that during the proliferative and early secretory phases, PGR-B and PGR-A are evenly distributed in the nucleus of positive cells. During the mid-secretory phase, PGR is less evenly distributed in the nucleus, rather sequestering PGR into foci within the nucleus. In the late secretory phase, the foci are even more pronounced, with a further decrease in nuclei with an even distribution of PGR over proliferative nuclei. The PGR-B isoform is consistently more sequestered into foci than PGR-A (Arnett-Mansfield et al., 2004). This is the first evidence that PGR may not be evenly distributed in the nucleus and the difference in foci formation between isoforms suggests that subnuclear distribution may be implicated in isoform function or regulation.

A caveat to immunoblotting and IHC using an antibody directed to a common region of PGR-B and PGR-A is that different antibodies have markedly different efficacies of isoform detection. Six out of 10 commercially-available antibodies assessed were not able to detect PGR-B in IHC, whereas they were able to do so in Western blot analysis on samples treated with standard antigen-retrieval techniques using boiling in 0.01 M citrate buffer (Mote et al., 2001).

The PGR expression in myometrium of pregnant women was assessed by Western blot and IHC at a pre-term stage of gestation, at term and during labor (Merlino et al., 2007). The PGR-A isoform was greatest at labor, with a decreased expression at

term and the least abundance in the pre-term myometrium, detected by Western blot. Conversely, PGR-B was most abundant pre-term. Using a PGR-A/B antibody for IHC, total PGR was greatest at labor, decreased at term and further decreased in pre-term samples, suggesting that PGR-A is the predominant form of PGR expressed in the pregnant myometrium (Merlino et al., 2007).

Non-human primates have provided a valuable resource in studying PGR protein expression, as well. In baboons, Western blots and IHC using a PGR-A/B antibody, a decrease in PGR-A/B in the GE of endometria with simulated endometriosis (Jackson et al., 2007) was shown. Neither PGR-B nor PGR-A were detectable in the stroma of affected endometria but were detectable in the stroma of control animals (Jackson et al., 2007).

Two studies examined PGR expression in marmoset endometrial cells (Silvestri et al., 2007; Narkar et al., 2006). Using an antibody to assay PGR-A/B, PGR was increased during the mid-late proliferative phase of the menstrual cycle and was least expressed during the early secretory phase, with the greatest differences subjectively observed in the LE. Interestingly, the antibody was directed to detect both PGR-B and PGR-A, but it was unable to detect PGR-B in the myometrium only (Silvestri et al., 2007). This suggests that either PGR-B is absent or undetectable in the myometrium or that protein folding in this specific cell type prevents reactivity with the PGR-directed antibody. Likewise, in the second study, a PGR-A specific antibody was used for IHC to detect low, constitutive expression of PGR-A in the stroma throughout the menstrual cycle (Narkar et al., 2006). In the GE, PGR-A was highly expressed in the early follicular phase, decreased in the late follicular phase, very weakly expressed in the mid-luteal

phase and expression was recovered in the late luteal phase (Narkar et al., 2006). In humans, PGR persists through the late follicular phase, decreasing after P4 begins to be secreted by the luteinized follicle, demonstrating another species-specific pattern of expression (Mote et al., 1999; Tseng and Zhu, 1997). The finding suggests that PGR-A expression may not be under the primary control of E2 in marmosets.

Visualization of PGR in mouse uterine LE using a PGR-A/B probe was performed by IHC (Kurita et al., 2000a; Kurita et al., 2001). Expression of total PGR was strong in the LE and GE of wild-type, ERKO, and ovariectomized plus E2 treated mice. In the wild-type uterus, E2 presence decreased PGR expression in the LE and GE and P4 increased PGR expression in the stroma. Treatment of ovariectomized mice with E2 increased PGR in both the vaginal epithelium and stroma in wild-type mice. Treatment of wild-type, ovariectomized mice with E2 significantly increased vaginal epithelium PGR expression over non-treated samples, while decreasing PGR expression in uterine LE versus non-treated samples (Kurita et al., 2000a). In ERKO mice, IHC with a PGR-A/B antibody detected strong PGR expression in the uterine LE and adjacent stroma with low to absent expression in distal stroma and myometrium. When animals were supplemented with E2, no detectable change in expression occurred in the LE, while PGR abundance increased in the stroma and myometrium (Kurita et al., 2001).

The oviduct and uterus of prepubertal mice were assessed for PGR protein expression using Western blot and IHC (Shao et al., 2006; Zeng et al., 2008; Aupperlee, 2007). Using a PGR-A/B antibody, PGR-B was detected at 115 kDa in both the oviduct and uterus. PGR-A was detected as a bright band at 83 kDa in both the oviduct and uterus; however, a less intense, slightly lower molecular weight band was present in the

oviductal sample, and two less intense bands, one of slightly higher and one of slightly lower molecular weight were apparent in the representative Western blots. Using manual assessment of Western blot and IHC results, a time-dependent decrease in PGR-B, PGR-A and total PGR was observed in both the oviduct and uterus in response to a single P4 injection (Shao et al., 2006).

A Western blot analysis of pregnant mouse myometrium with a PGR-A/B antibody detected PGR-A at 81 kDa, but was unable to detect PGR-B. Analysis by IHC demonstrated that PGR-A was more highly abundant at 17.5 dpc than 19.5 dpc (Zeng et al., 2008). This provides another example of an antibody unable to detect one isoform of PGR, suggesting that protein folding and antigen availability may be quite distinct between PGR-B and PGR-A.

Finally, the mammary glands of virgin adult ovariectomized mice were assessed by IHC using a PGR-B specific antibody and a PGR-A specific antibody (Aupperlee, 2007). The PGR-A specific antibody was deemed specific based upon empirical evidence that it was consistently unable to detect PGR-B in Western blot analyses. Specificity to PGR-A protein, therefore, was not due to recognition of an antigen sequence specific to PGR-A. The PGR-B isoform was undetectable in mammary tissue immediately after ovariecetomy even when stimulated with estrogen. The PGR-B expression increases slightly to a very low presence on day 5 after ovariectomy in response to both E2 and P4 treatment and by d 10 in response to P4 treatment alone. The PGR-B isoform was only reliably detected after d 10, with approximately 25% of epithelial cells PGR-B positive. Expression of PGR-A was decreased in ovariectomized mice, but this loss was immediately attenuated in response to E2 supplementation. When

E2 was supplemented in conjunction with P4, however, PGR-A protein expression decreased to less than that of untreated animals. Additionally, proliferation activity was assessed using BrdU staining. Nearly half (49%) of the BrdU positive cells were also positive for PGR-B, whereas only 3% were positive for PGR-A, indicating that PGR-B is the isoform responsible for proliferative activity of epithelial cells, as anticipated (Aupperlee, 2007).

In rats, PGR abundance in the endometrium was assessed at the protein level in response to E2 stimulation. Using Western blots to assay protein abundance, Kraus and Katzenellenbogen (1993) demonstrated a time- and dose- dependent transient up-regulation of PGR in response to E2 stimulation. Protein abundance reached its peak with a 6-fold increase in expression 48 hours after E2 stimulation. Treatment with P4 was sufficient to block E2-mediated up-regulation in protein PGR abundance (Kraus and Katzenellenbogen, 1993).

Using PGR-B and PGR-A/B antibodies on uterine tissue sections from ovariectomized rats supplemented with either oil (control) or E2 as a hormone replacement therapy (Ilenchuk and Walters, 1987) was analyzed by immunohistochemistry for PGR expression. Treatment with E2 increased PGR-B and PGR-A/B in both the stroma and myometrium over control animals significantly. Treatment with E2 decreased both PGR-B and PGR-A/B, however, in the LE over control animals. Time between E2 injection and tissue fixation may account for the unexpected decrease in both PGR isoforms in the LE. The ratio of PGR-B to PGR-A in the rat uterus, determined by photoaffinity binding assays, was observed to be very similar, with approximately equal concentrations of both isoforms (Ilenchuk and Walters,

1987).

In the endometrium of Nelore cattle, an antibody directed to PGR-A/B was used for IHC and showed nuclear rather than cytoplasmic staining (Vermeirsch et al., 2002). The percentage of PGR-A/B positive nuclei did not vary significantly in either the GE or stroma throughout the estrous cycle. Percent positive nuclei, however, was greater in the stroma than in the GE. In a study of the canine oviduct, IHC detected PGR-A/B at increased abundance during proestrus and decreased during early metestrus, while stroma staining was consistently more intense than in the epithelial cells (Vermeirsch et al., 2002). The study also found that ERα was expressed at a high abundance during proestrus. Both of these steroid receptors showed nuclear staining. Androgen receptor (AR), however, was observed to be largely cytosolic throughout the cycle in this tissue (Vermeirsch et al., 2002). These results show an expected pattern of total PGR and ERα expression in the endometrium. The results also establish the presence of a cytosolic nuclear receptor superfamily member. Expression of other superfamily members, ER and PGR was observed in the endometrium of women throughout all phases of the menstrual cycle (Berkquist et al., 1981). In a study of total PGR, expression was observed to be cytosolic in the human ovary, as well (Jacobs et al., 1980). In the endometrium of the sea lion (Zalophus californianus), IHC detected little variation during the estrous cycle in total PGR. PGR-A/B was also detected in the vaginal epithelium and stroma as well as the cervix with little change throughout the estrous cycle. In all tissues, however, the stroma stained more intensely than the epithelium (Colegrove et al., 2009). These results demonstrate a similar pattern of PGR-A/B expression in disparate mammalian species. Again, the inability to distinguish between cell-type specific and isoform-specific

expression may inhibit the potential of these studies to detect changes in PGR in conjunction with the estrous cycle.

Human and rat non-reproductive tissues have also been assessed for PGR expression. In a study of human meningiomas, IHC with a total PGR antibody was used to confirm Northern blot analysis of PGR presence. Assays using ICC detected intense nuclear staining and little to no cytosolic staining in positive cells. However, only 6 of 11 sections positive for PGR at the mRNA level were positive for PGR at the protein level (Carroll et al., 1993). This may indicate an unavailability of the PGR antigen in these samples to the antibody or may again suggest an uncoupling between PGR mRNA abundance and protein abundance. In a study of PGR expression in rat vertebral cell populations, 2 antibodies common to PGR-A/B were used for Western blot and IHC procedures (Pei et al., 2006). Molecular weight of PGR-B was detected at 116 kDa by Northern Blot, and PGR-A was detected at 81 kDa. Only PGR-A, however, was able to be consistently detected by either antibody, with PGR-B often undetectable even in positive control samples. The IHC of two cohorts of rats aged less than 19 months and 3 cohorts of rats aged greater than 20 months indicated that P4 and E2 are able to work synergistically to increase total PGR only in rats aged less than 19 months, while the two steroid hormones increased specifically PGR-A at all ages as detected by Western blot (Pei et al., 2006). This study demonstrates the caveats involved in IHC without Western blot validation of antibodies in specific sample populations and also the temporal-specific ability of steroid hormones to regulate PGR protein expression.

GENETIC ENGINEERING

The availability of genetic engineering technology has facilitated the discovery of the divergent activities of PGR-B and PGR-A isoforms. The "knockdown" method of protein disruption using small interfering RNA (siRNA) to target PGR-B or PGR-A RNA for degradation before translation has been implemented *in vitro* in human endometrial and myometrial cells, as well as the breast carcinoma cell line T47D (Hardy et al., 2006; Wu et al., 2008; Brayman et al., 2006;). The "knockout" method of gene disruption via homologous recombination has been used to selectively study the abrogation of either PGR-B or PGR-A specifically, both *in vitro* using a human LE cell line and *in vivo* using a whole animal mouse model.

In the absence of both PGR-B and PGR-A targeted by siRNA knockdown, basal levels and interleukin-1β (IL-1β)-induced cyclooxygenase-2 (Cox-2) were increased despite the presence of P4 in human myometrial and T47D cells (Hardy et al., 2006). Expression of PGR-B alone was unable to repress the activation of Cox-2, but PGR-A alone was able to act as an anti-inflammatory in the presence of P4 to block transcription of Cox-2 (Hardy et al., 2006). In the YHES human endometrial stroma cell line, PGR-B was knocked down using siRNA. When the amount of PGR-B was decreased by 50%, cell proliferation was increased by 20% which was significant over mismatch siRNA and over untreated control cells (Wu et al., 2008).

In HES cells devoid of PGR-A, PGR-B mediates a P4 induction of mucin-1 (MUC1) transcription via a PRE in the promoter of MUC1 (Wu et al., 2008). This induction was antagonized in cells expressing PGR-A (Wu et al., 2008). Using site-

directed mutagenesis of PGR-B and PGR-A domain C (DNA binding domain), Brayman et al. (2006) demonstrated PGR-B acts as an activator via the DBD, while the DBD is not essential for PGR-A repression of PGR-B activation. ChIP assays demonstrated that either PGR-B or PGR-A may be recruited to the PRE in the MUC1 promoter, but less binding was observed in the presence of both PGR-B and PGR-A. In a series of complementary experiments in knockout mice, wild type ovariectomized mice lost expression of MUC1 in the LE and GE (Brayman et al., 2006). Expression was rescued by E2 supplementation and rescue was attenuated with E2 plus P4 supplementation. In total PGR knockout (PRKO) mice, MUC1 was not lost from the LE and GE due to ovariectomy, but was stimulated by both E2 and E2 plus P4 treatment. The phenotype of the PGR-B knockout mice (PRBKO) mimicked the wild type phenotype, while PGR-A knockout mice (PRAKO) mimicked the PRKO phenotype, indicating that PGR-A is responsible mediating the activation of MUC1 by P4 (Brayman et al., 2006). Taken together, these results suggest PGR-B and PGR-A may have different activities in the same cell type of different species or that PGR acts differently in cell culture than *in vivo*.

Knock-out mouse models have been useful for further characterization of the interaction between ERs, P4 and PGR expression. An ERAKO mouse was used to investigate the importance of the specific estrogen receptor on PGR regulation (Kurita, 2000a). In mice, estrogen has the opposite effect upon PGR than in other mammalian species, namely that E2 down-regulates PGR. Using knockout models, ERα was demonstrated to be necessary and sufficient for E2 to mediate its down-regulation in murine endometrial LE and stroma, though constitutive expression of PGR was not altered (Kurita, 2000a). Cultured endometrial tissue of wild-type stroma with ERAKO

LE and ERAKO stroma with wt LE demonstrated, when grafted into nude mice, that functional ERα in the stroma was necessary and sufficient for down-regulation of PGR in the LE, implicating communication between endometrial cell types as an important mediator of E2 (Kurita, 2000a).

Using PGR knockout mouse models, Mulac-Jericevic et al. (2000) were able to determine distinct biological functions in the mouse uterus for PGR-B and PGR-A isoforms. In PRAKO mice, increased branching of mammary alveolar tissue was observed that was not present in wild-type of PRKO mice. When exogenous E2 is administered alone, the LE of wild-type, PRKO and PRAKO mice was induced to proliferate rapidly. In wild-type mice, the presence of exogenous P4 abrogates the hyperplasia of LE cells. A similar inhibition of proliferation was observed in PRKO mice. In PRAKO mice, however, proliferation was increased in the presence of both P4 and E2. Taken together, these results demonstrate distinct biological functions for PGR-B and PGR-A isoforms in the endometrium of mice, with PGR-A activity responsible for increased cell proliferation and PGR-B acting to attenuate that proliferation (Mulac-Jericevic et al., 2000).

To examine the tissue-specific expression of PGR in the endometrium, tissue recombinants of wild-type and PRKO endometrium were constructed by isolating the stroma or LE from one treatment and culturing with the LE or stroma, respectively, of the other treatment (Kurita, 2000b). The recombined endometrium was then grafted into a nude mouse. Exogenous E2 was able to down-regulate PGR in both wild-type stroma with PRKO LE and in PRKO stroma with wild-type LE. Down-regulation of PGR stimulated by E2 in wild-type animals, however, was only able to be blocked by P4.

Abundance of PGR was measured at the mRNA and protein levels. Thus, unlike ERα, PGR must be present in both the stroma and the LE to mediate the P4 inhibition of E2 down-regulation in the mouse (Kurita, 2000b).

In mouse mammary tissue, PRKO mice display decreased branching of ducts and cell proliferation (Ismail et al, 2003). Implantation of embryos is impossible in PRKO mice. The PRBKO mice support implantation and inhibit unrestrained proliferation of epithelial tissue. The PRAKO mice experience high proliferation of LE, but uterine cells do not differentiate to become decidualized (Conneely et al., 2003). Complementary experiments in the uteri of PRAKO and PRBKO mice demonstrated only PGR-B expressed in the LE with both PGR-B and PGR-A expressed in the stroma and myometrium and only weak PGR-A in the GE. PGR-B expression did not vary with stage of estrous cycle, while PGR-A was most abundant on the morning of proestrus and least abundant on the evening of proestrus and was constitutively expressed highly in the myometrium (Mote et al., 2006). Both PGR-B and PGR-A expressed in the hypothalamus also have an effect on sexual behavior. PRAKO sexually mature females display almost no lordosis response to males (Mani et al., 2006). PRKO and PRBKO sexually mature females demonstrated an increase in challenge behavior, such as biting, to males during the putative receptive period, while aggression of wild type males to these genotypes was also increased (White et al., 2007).

Another method of investigating the activity of either PGR-B or PGR-A specifically is by ectopic expression of either the B or A isoform using a transfection vector or the "knockin" method. This method is also used either *in vitro* in human cell lines or *in vivo* using a whole animal mouse model. In a breast carcinoma cell line,

HeLa, a model was developed in which a reporter construct under the control of 3 EREs in the promoter region was cotransfected with overexpression of ERα and either PGR-B or PGR-A. When PGR-A is overexpressed in combination with overexpression of ERα, reporter gene expression was inhibited. This inhibition was relieved but not completely recovered by the antagonistic activity of PGR-B. These results were confirmed in a hepatic carcinoma cell line, HepG2. The results of the study indicate that PGR-B and PGR-A may act through different coactivators, with PGR-B being in direct competition with ERα for a common activator (Giangrande et al., 2000).

Another HeLa model uses a PRE-driven thymidine kinase CAT reporter construct transfected with either PGR-B or PGR-A to determine efficacy of the isoforms in driving transcription via PREs in the presence of the NF-κB subunit RelA(p65), a mutual repressor (Kalkhoven et al., 1996). The RelA(p65) subunit was able to repress PGR-B and PGR-A transcription factor activity in the presence of P4, although PGR-A was consistently less effective at activating the reporter gene. However, PGR-B and PGR-A both repressed RelA(p65) transcription factor activity with or without the presence of P4. These results were confirmed in the monkey cell line COS-1 and breast cell carcinoma cell line T47D which expresses both PGR isoforms endogenously. Using deletion constructs, Kalkhoven et al. (1996), demonstrated that the C domain responsible for DNA binding was essential for repressive activity, while ablation of either activation factor (AF-1 or -2) did not affect repression of RelA(p65; Kalkhoven et al., 1996).

In an ER-negative breast cancer cell line, MDA-MB-231, Leo and Lin (2008) demonstrated that while P4 mediates the effects of decreased DNA synthesis and increase in cell spreading in culture via either PGR-B or PGR-A, P4 antagonist RU486 acts

differently, inducing cell spreading in PGR-B transfected cells and alterations in cell morphology in the presence of overexpressed PGR-A (Leo and Lin, 2008). Similarly, in TE671 medullablastoma cell line, P4 decreases the transcription of the GnRHR1 promoter in PGR-A transfected cells by 3 fold, while increasing GnRHR1 transcriptional activity by 4 fold in the presence of overexpressed PGR-B (An et al., 2005). In the presence of P4, both PGR-B and PGR-A are capable of inhibiting the prolactin-mediated transcriptional activation of β-casein in cultured murine mammary epithelial cells, while PGR-B is more efficacious, reducing reporter gene expression by 65% (Buser et al., 2007).

In cultured human endometrial cells, P4 mediated a decrease of metallomatrix proteinase 2 (MMP2) expression via the PGR-B isoform, specifically, but increased pro-MMP2 expression when either PGR-A or PGR-C were transfected. However, while PGR-A in the absence of P4 does not alter MMP2 promoter activity, PGR-C is capable of decreasing promoter activity in the absence of its ligand, suggesting PGR-A is capable of repressing the activity of PGR-B, while PGR-C is capable of repressing both PGR-B and PGR-A (Goldman and Shalev, 2007). Also in human endometrial stromal cells, PGR-A is more efficacious in activating the insulin-like growth factor binding protein-1 (IGFBP-1) via 2 PREs than transfected PGR-B. PGR-A activates in a dose-dependent fashion. PGR-B also reduces the activity of PGR-A when co-transfected in a dose-dependent manner, decreasing IGFBP-1 transcription by 50% and 60% when PGR-B/PGR-A was 0.3 and 1, respectively (Gao et al., 2000).

Transgenic mice were created which expressed additional PGR-A, increasing the natural, endogenous ratio of PGR-A/PGR-B, resulting in a whole animal model of PGR-

A overexpression to study endometrial pathology (Fleisch et al., 2009). The uteri of these mice demonstrated an increase in the proliferation of luminal epithelial cells. This increase was more pronounced in the presence of P4, and in the presence of both E2 and P4, the hyperplasia of luminal epithelial cells appeared to be unrestrained, suggesting that ratio of the PGR isoforms is essential for normal uterine physiology and implicates an imbalance in this ratio as a potential cause of pathology (Fleisch et al., 2009). These results are consistent with the non-classical response of PGR to steroid hormones in the mouse, although they appear to be in contrast with other species in which identify PGR-B is the primary isoform responsible for proliferation, and PGR-A is the primary isoform responsible for attenuating this proliferation, so it is possible that imbalance of the PGR-A/PGR-B ratio does not simply increase the activity of PGR-A over the activity of PGR-B, but results in unanticipated and more complex interactions.

CANCER

The PGR plays integral roles in human uterine and breast cancer biology. Thus, significant research exists on expression and regulation of PGR at the mRNA and protein levels in these tissues. Estrogen-responsive human breast cancer cell lines T47D and MCF-7 have been studied to investigate PGR dynamics at the protein and mRNA levels in mammary tissue. In T47D cells, only the PGR-B but not the PGR-A protein isoform was up-regulated in response to treatment with E2 (Graham, 1995). The PGR-B promoter was induced by E2 and thus, PGR-B transcripts. Progesterone treatment was

sufficient to ablate E2 up-regulation of PGR-B at the levels of mRNA and protein, but did not have any effect upon the activity of either the PGR-B or the PGR-A promoter to directly alter transcription rates (Graham, 1995). In both T47D and MCF-7 cells, five different transcripts have been identified, with one primary transcript (Read et al, 1988). The PGR mRNA and protein expression was assessed by Northern and Western blots, respectively in control and P4 treated T47D cells, and PGR abundance (both mRNA and protein) was decreased by 90% in response to P4 stimulation. In MCF-7 cells, when cells were cultured in the absence of E2, PGR mRNA and protein were undetectable by Northern and Western blots. However, when cultured in the presence of E2, a proportionate increase in abundance of mRNA and protein was observed (Read et al, 1988). Further characterization of PGR profiles in T47D and MCF-7 cells was conducted by Wei et al (1988). At basal levels, the authors estimate that T47D cells contain 90 PGR mRNA molecules per cell with or without E2 treatment. In MCF-7 cells, the basal level is 16 PGR mRNAs per cell, up-regulated to 45 molecules when the cells are treated with E2. When T47D cells are treated with progesterone, mRNA and protein of both PGR-B and PGR-A were transiently down-regulated for 8 to 12 hours and refracted to prior abundance during the subsequent 48 hours, supporting the hypothesis that PGR directly inhibits its own transcription and translation by autoregulation (Wei, 1988). Regulation of PGR by other mediators may not be as straightforward. The MCF-7 cells respond to E2 stimulation by increasing PGR mRNA, and subsequently protein. In the same cell line, however, IGF-I mediates up-regulation of PGR by inducing an increase in protein abundance without concomitant mRNA increase. Also, E2 and IGF-I mediate their effects differently in the presence of serum in cell culture, inhibiting the up-regulation of

IGF-I while enhancing the increased expression mediated by E2. The two stimulators of PGR expression, E2 and IGF-I, therefore mediate their effects through different molecular mechanisms (Cho, 1994). Contradictory evidence from 2002 indicates that IGF-I decreases PGR mRNA and protein expression in the same (MCF-1) cell line. The study maintains that while P4 targets PGR protein for degradation by the proteasome, IGF-I acts at the level of PGR transcription to down-regulate expression, acting through the PI3 kinase pathway (Cui et al., 2003). These contradictory results emphasize the variability in PGR expression in different cell types and perhaps the dangers of conducting experiments with immortalized cell lines in vitro. The most effective way to solve the dispute may be to investigate the action of IGF-I *in vivo*.

Finally, studies have focused on the expression of the PGR protein only. To study PGR protein expression in the uterus, human primary uterine cell culture and baboon endometrium have been examined (Aronica and Katzenellenbogen, 1991; Jackson et al., 2007). To investigate the PGR protein patterns in breast tissue, murine mammary gland tissue as well as human breast cancer cell line MCF-7 was used. The kinetics of PGR hormone binding and turnover have also been examined in estrogen-receptor positive MCF-7 cells. Molecules known to up-regulate transcription of PGR were studied in human uterus cell culture. Estrogen, IGF-I and cyclic adenosine monophosphate (cAMP) were supplemented to cell culture and PGR protein expression was monitored by Western blot. Both molecules induced an up-regulation of PGR protein, although the antibody used did not differentiate between PGR-B and PGR-A. Binding capability of PGR was also assessed using a radiolabeled progesterone agonist [3H]R5020 and was increased in response to all three agents, verifying that functional PGR protein was produced.

Inhibition of E2, IGF-I and cAMP by ICI 164, or protein kinase inhibitors H8 or PKI, respectively resulted in no up-regulation of PGR protein expression (Aronica and Katzenellenbogen, 1991). While these results agree with transcriptional control described in the uterus, PGR expression may be cell-type specific, age-dependent (Kohler et al., 2007), or even specific to the stage of the cell cycle (Narayanan et al., 2005). Studies show loss of PGR from the LE of the uterus in pigs and other species during the normal estrous cycle (Tseng and Zhu, 1997; Geisert et al, 1994; Sukjumlong et al., 2005; Ross et al., 2010), but PGR expression in the stroma is not ablated. In baboon models for endometriosis, Jackson et al. (2007) demonstrated PGR-B and PGR-A protein expression in baboon endometrium using Western blots and immunohistochemistry. Expectedly, both PGR-B and PGR-A proteins were down-regulated in the GE. In the stroma, however, total PGR protein was not lost. While treatment with estrogen increased the protein abundance of PGR-B and PGR-A in normal baboon endometrium, the treatment had no effect on protein expression in animals with the endometriosis phenotype. Thus, endometriosis disrupts estrogen effects on PGR isoform expression exclusively in the stroma of baboons, demonstrating a disease-specific and cell-type-specific expression pattern (Jackson et al., 2007).

Indeed, in murine mammary tissue, estrogen up-regulation is specific to PGR-A protein, while PGR-B expression is increased by either P4 alone or P4 and E2 (Aupperlee and Haslam, 2007). Assessed using PGR-B and PGR-A specific antibodies for Western blots, dominance of PGR-B isoform or PGR-A isoform also had distinct biological consequences, with PGR-B stimulating alveologenesis and PGR-A inducing cell proliferation (Aupperlee and Haslam, 2007). While the agreement between studies

indicating transcriptional up-regulation of PGR expression by E2 and increased protein abundance imply that increased PGR protein is due to increased synthesis, attenuated degradation of PGR protein may also play a role in E2-induced PGR protein increases. In estrogen receptor positive MCF-7 cells, dense amino acid incorporation into nascent protein chains was used to address this question. Thus, PGR degradation rates were estimated to be equivalent whether at half-maximal (3 X 10 (-11) M E2) or maximal (6 X 10 (-11) M E2) expression of PGR due to E2 stimulation. Since PGR degradation is not decreased in response to maximal expression from E2 stimulation, it can be inferred that increased PGR protein expression is due to increased synthesis (Nardulli, 1988). Conversely, another study utilized the same method of dense amino acid incorporation to investigate protein synthesis and degradation of PGR in response to P4 in MCF-7 cells (Mullick and Katzenellenbogen, 1986). Using a sucrose gradient shift analysis, Mullick and Katzenellenbogen (1986) calculated the half-life of PGR protein in the absence of P4 down-regulation to be 17 hours. The turnover rate in the presence of a radiolabeled P4 was increased, resulting in a half-life of only 12 hours. By running the [3H]R5020 bound ligand on an SDS-PAGE gel, the authors determined that both PGR-A and PGR-B turnover rates were increased in the presence of progestins (Mullick and Katzenellenbogen, 1986). In a separate analysis of T47D cells, protein turnover rates were also assessed in response to P4 treatment. This study found that the half-life of PGR in control cells was 21 hours, decreasing to only 6 hours when cells are treated with P4. Both PGR-B and PGR-A were affected equally by P4 in increasing receptor degradation (Nardulli and Katzenellenbogen, 1988). Thus, while estrogen increases

transcription and synthesis of PGR isoforms, P4-stimulation results in increased protein degradation of bound receptors.

PROGESTERONE RECEPTOR IN THE PIG

Expression of the PGR transcript has been studied previously in porcine cumulus cells, pituitary and hypothalamus regions of the brain, oviduct and whole uterine tissue (Shimada et al., 2004; Diekman and Anderson, 1983; Slomczynska et al., 2000; Peralta et al., 2005; Tummaruk et al., 2009; Sukjumlong et al., 2005). Porcine cumulus-oocyte complexes (COCs) collected from a slaughterhouse were matured in vitro and assessed at the mRNA and protein levels (Shimada et al., 2004). Using qRT-PCR, neither PGR-B nor PGR-AB expression was increased by culture with E2 alone, but were increased by FSH ($P < 0.01$). When cultured for an extended period of time with FSH and LH, PGR-B mRNA increased up to 8 hours, and then was down-regulated in abundance by 12 hours in culture. PGR-AB increased continually to the endpoint of 20 hours in culture, suggesting PGR-B mRNA transiently responds first to gonadotropins, with a lagging and possibly sustained up-regulation of PGR-AB transcription. Protein abundance of PGR was assessed using immunoblotting with a PGR-A/B antibody. The PGR-B isoform was detected at 116 kDa, PGR-A was detected at 97 kDa, and a third isoform was detected at 66 kDa identified as PGR-C. When cultures were stimulated with gonadotopins FSH and

LH, PGR-B, expression increased from hours 4 through 12, then decreased to a low at 20 hours. Expression of PGR-A increased throughout treatment, with the greatest abundance detected at the final time point assessed, 20 hours. PGR-B was consistently the predominant form of PGR protein expressed, and expression of PGR-C was not assessed in treatment samples (Shimada et al., 2004). There was a short lag of approximately 4 hours between up-regulation of PGR-B transcript and subsequent up-regulation of PGR-B protein; however down-regulation of both was seen at the same time, suggesting that PGR-B protein might be actively degraded, rather than simply transcription decreased. The PGR-AB isoform did not exhibit the lag between increase in transcription and increase in protein, and both continued to increase over the entire period examined.

Porcine pituitary and hypothalamus glands collected from cyclic pigs on days 1, 5, 10, 15 and 18 and from pregnant pigs on days 5, 10, 15, 21, and 30 after mating and whole tissues flash frozen and stored (Diekman and Anderson, 1983). Presence of PGR protein was assessed in the tissue in both the nuclear and cytosolic fractions using a competitive binding assay. There were no statistical differences in the cytoplasm of cyclic pigs between days 1 – 15. On day 18, however, PGR was significantly decreased. Nuclear expression of PGR protein was increased on day 1 over days 5 and 18. In the hypothalamic tissue, cytosolic PGR protein expression was increased on day 15 over all other days analyzed and nuclear PGR protein expression was greater on day 18 over all other days analyzed. Neither cytosol nor nuclear expression of PGR varied throughout days of early pregnancy in either the hypothalamus or pituitary glands (Diekman and Anderson, 1983). The observed pattern of expression is consistent with the classical

mechanism of PGR being down-regulated by high, sustained presence of its ligand, P4. This is also consistent with Ross et al. (2007) data indicating that PGR expression is similarly expressed both in pigs that are in early pregnancy and cyclic pigs.

Porcine ovaries were collected from a slaughterhouse and developmental stage classified as small, medium or large diameter follicles. Corpora lutea were removed and classified as newly-formed, functional or regressing (1, 2 or 3; Slomczynska et al., 2000). Using a PGR-A/B antibody, Western Blots detected porcine PGR-B at 120 kDa and PGR-A at 86 kDa. Subsequent IHC detected that granulosa cells from small and medium follicles were negative for PGR. Theca cells of the same follicles were all positive. The granulosa cells of preovulatory large follicles and stage 1 corpora lutea (CL) were also positive for PGR. Treatment of follicles with gonadotropins FSH and LH increased the expression of PGR in granulosa cells. In the corpora lutea, almost all cells were PGR positive, however, the amount of expression declined during luteal regression. PGR expression was strictly nuclear in all PGR positive cells (Slomczynska et al., 2000).

Porcine PGR-B and PGR-A both appear at slightly increased molecular weights than the isoforms in humans, mice or rats (Slomczynska et al., 2000; Shimada et al., 2004; Harduf et al, 2009; Shao et al., 2006; Kraus and Katzenellenbogen, 1993). This may be due to increased covalent modifications to porcine PGR or may be an artifact of the experimental technique. Interestingly, a study to detect PGR in porcine sperm membrane proteins using a Western Blot with a PGR-A/B antibody detected only 2 isoforms migrating to 74 kDa and 63 kDa (Wu et al., 2006). This may indicate degradation of the protein when the extracted sperm membrane proteins are subjected to an affinity column. It may also indicate additional PGR isoforms that are tissue-specific

and have not been encountered in porcine female reproductive tissues.

In porcine oviduct, using a common PGR-A/B probe designed to the ligand binding domain of the PGR protein, mRNA abundance detected by qRT-PCR remained constant throughout the follicular and luteal phases of the estrous cycle (Peralta et al., 2005). This may be due to a mixture of cell types analyzed for PGR, as cell-specific expression is likely based upon evidence in other tissues and species. Total PGR protein, however, varied during the cycle in the ampulla and isthmus regions of the oviduct. Using IHC with a PGR-A/B common antibody, total PGR in the ampulla was low in the stroma in the follicular phase and increased in both the LE and stroma during the luteal phase. In the isthmus, PGR was undetectable during the follicular phase and increased in both the LE and stroma during the luteal phase. LE cells in both the ampulla and isthmus consistently had an increased percentage of PGR positive nuclei (33 to 65%) than the stroma (11 to 33%). Finally a competitive binding assay was used to determine functionality of PGR in the oviduct. The cytosolic fraction during the luteal phase showed the least binding capacity, with an increased capacity in the nuclear fraction during the luteal phase, followed by an increased capacity in the cytosolic fraction during the follicular phase, and the nuclear fraction during the follicular phase demonstrating the greatest PGR binding capacity (Peralta et al., 2005). Again, these results demonstrate an uncoupling of mRNA level and protein level of total PGR in the pig. Additionally, Peralta et al. (2005) suggest functional PGR protein presence in the cytosolic fraction of cells.

The utero-tubal junction (UTJ) of 15 multiparous sows was assessed from PGR protein expression using IHC with a PGR-A/B antibody (Tummaruk et al., 2009). Sows

were randomly assigned to artificial insemination (AI), intra-uterine insemination (IUI), deep intra-uterine insemination (DIUI) or no artificial insemination treatments (NAI) and were hysterectomized 24 hours after insemination. PGR expression was examined as a mediator of sperm and embryo transport induced by P4. Total PGR expression was similar in the surface and deep GE and LE of the UTJ region of the oviduct in all treatment groups. However, PGR expression in the stroma and myometrium of NAI sows was significantly less abundant than in AI and IUI treatment groups. A proposed theory is that the E2 present in semen samples induced PGR expression in the UTJ to prepare for sperm and embryo transport. However, DIUI uses a significantly reduced amount of semen compared to both AI and IUI, which would result in less E2 and perhaps less PGR induction over control tissue (Tummaruk et al., 2009). Interestingly, PGR up-regulation was demonstrated only in the stroma and myometrium, the tissue most distal to the proposed semen E2 mediator of PGR expression. This implicates effective cross-talk among the different cell types of the endometrium and demonstrates cell-specific regulation of PGR by the same stimulus.

A study of Landrace x Yorkshire multiparous sows at 5 stages of cyclicity (d 1, d4, d 11-12, d 17 and d 19, n = 3 per treatment, d 1 is 12 hours after standing estrus detection) and 5 stages of early pregnancy (d 1, d2, d4, d 11, d 19, n = 3 to 4 per treatment, d 1 is 5 to 6 hours after artificial insemination) used a cDNA probe designed to the ligand binding domain of human PGR for qRT-PCR to detect PGR-A/B expression at these stages in both the myometrium and the endometrium (Sukjumlong et al., 2009). In the endometrium, PGR-A/B mRNA was lowly expressed at all stages when compared to ERα expression. Expression fluctuated during the cycle, however, with significantly

increased abundance at early diestrus (d4) than mid or late diestrus (d 11 – 12, d 17). For sows in early pregnancy, PGR-A/B was most abundant on d4, significantly increased over d 11 or d 19 expression with intermediate transcript abundance on days 1 and 2, suggesting that the endometrium follows a similar pattern of mRNA expression regardless of pregnancy or cyclic status. In the myometrium, PGR-A/B mRNA in cyclic animals was significantly increased in abundance during proestrus (d 19) over all other days. For sows in early pregnancy, PGR-A/B was more abundant on d 1 immediately following insemination than any other time points (Sukjumlong et al., 2009). This study does not differentiate between PGR isoforms, so isoform-specific and cell-type-specific expression within endometrium may be undetectable in this study. These results do indicate, however, that PGR in the endometrium of pigs responds in a classical manner to E2 and P4 stimulation, namely increased by E2 and decreased by prolonged exposure to P4. They also suggest that pigs exhibit tissue-specific expression, as well. In a parallel study of the same treatment groups, IHC with a PGR-A/B antibody was used to assay total PGR protein expression in the porcine uterus. In the LE, cyclic animals had high expression of total PGR at estrus and early diestrus. Early pregnant animals had greater PGR expression on d 1 and d4 compared to d2, d 11 and d 19. In the GE, cyclic animals almost all cells were positive for PGR at proestrus and estrus, with surface and deep glands significantly increased on d 1. Early pregnant animals had increased expression in the superficial glands on d4, and weak expression on d2 and d 19, while deep glands had high expression at d 1 and d2 with decreased expression on d 19 when E2 concentrations are low. In the stroma, all cells were PGR positive at all stages of cyclic animals with the most expression at estrus, significantly less at proestrus and early diestrus and still

significantly less at diestrus and late diestrus (Sukjumlong et al., 2005). This study does not differentiate between PGR isoforms, so isoform-specific expression in different cell types cannot be assessed. However, these results indicate that PGR in the endometrium of pigs responds in a classical manner to E2 and P4 stimulation, namely increased by E2 and decreased by prolonged exposure to P4.

In a study of 27 Large White x Landrace sows culled for infertility, IHC determined that PGR-A was absent from all uterine cell types, but could be detected in a positive control sample of porcine uterus at proestrus (Karveliene et al., 2007). This may indicate that PGR-A loss is either a contributing cause of anestrus or an effect of the anestrual state. Another possibility, once again, is that protein conformation may be altered, obscuring antibody binding in anestrus samples and that PGR-A is present but undetected. Finally, no accompanying Western Blot or description to explain the purported specificity of the PGR antibody used here to porcine PGR-A alone.

Pubertal crossbred gilts were hysterectomized on various days of cyclicity (day 0 = estrus, d 5, d 10, d 12, d 15, d 18; n = 3 to 4 per day) and early pregnancy (d 10, d 12, d 15, d 18; n = 3 to 4 per day; Geisert et al., 1994)). Western Blot with a PGR-A/B antibody detected PGR-B at a molecular weight of 117 kDa and PGR-A at 90 kDa on days 0 through 12 in cyclic animals. In cyclic animals, IHC detected nuclear total PGR in LE, GE and stroma. Expression of PGR in the LE and GE was greatest on d 5, steadily decreasing to undetectable amounts on d 12 and d 15. The deep GE and stroma had detectable expression throughout the cycle, and PGR expression in the myometrium of cyclic animals was constitutive on d 0 – d 18. Total PGR in pregnant animals steadily decreased from d 10 – d 18, with undetectable amounts on d 15 and d 18. Expression of

PGR on days 10 – 18 were not statistically significant between cyclic and pregnant animals. A competitive ligand binding assay of samples indicated high presence of active PGR on d 0 and d 5 of the estrous cycle, significantly decreasing by d 10. By d 12, functional PGR abundance was decreased to 25% that of d 5. Once again, there was no significant difference between functional PGR on d 10 to d 18 in cyclic and pregnant animals (Geisert et al., 1994). Western Blots performed on the cytosolic fraction of endometrial cells, showed, interestingly, the presence of both PGR-B and PGR-A in the cytosol. However, only nuclear staining was observed in IHC. This may indicate that PGR isoforms have a different conformation in the cytosol in which the antigen is accessible to the antibody probe after standard denaturation for Western blotting, but inaccessible even after standard antigen retrieval techniques in IHC.

CONCLUSIONS AND PURPOSE OF STUDY

Progesterone receptor transcription and translation in the uterus varies significantly between species and between cell types, as well as among physiological states. However, estrogen seems to act as a potent regulator (usually positive, rarely negative) of PGR transcription. This may be mediated by differential activator binding to induce either PGR-B or PGR-A isoform transcription. Binding of its ligand, P4, usually induces PGR protein turnover and prevents estrogen-induced up-regulation of PGR mRNA transcription. IGF-I and cAMP also appear to be key regulators of PGR expression. The PGR and its isoforms have not yet been characterized at the level of

DNA, mRNA or protein in the pig. Characterization of PGR and identification of isoforms present will facilitate investigation of PGR in the pig endometrium. Continued investigation of PGR dynamics in cell- and steroid hormone- specific conditions may help us to better understand the complex pattern of mRNA and protein expression of the different PGR isoforms in the pig.

CHAPTER III

SEQUENCE AND HOMOLOGY CHARACTERIZATION OF PORCINE PROGESTERONE RECEPTOR (PGR) MRNA AND PROTEIN ISOFORMS IN THE ENDOMETRIUM

ABSTRACT

The down-regulation of the PGR within the luminal epithelium enables conceptus attachment and signaling within the porcine uterus. In humans, three PGR mRNA isoforms (PGR-A, PGR-B, and PGR-C) arise from alternative transcription start sites. The mRNA isoforms encode proteins with truncated N-termini that may confer distinct biological functions. The objective of this study was to determine the nucleic acid and amino acid sequences of PGR isoforms found within the endometrium of the pig. Primer pairs for porcine PGR were developed from porcine genomic and human mRNA sequences and used to amplify porcine PGR fragments from cDNA. The porcine PGR mRNA sequence generated is 4.3 kb with 84% identity to human, 79% identity to mouse and 86% identity to cow PGR mRNA. The transcription initiation site for the full-length PGR transcript was determined to be located between 1 and 470 nucleotides. Three in-frame ORFs were identified and putative protein sequence inferred. The porcine PGR protein is 934 amino acids in length with 84% overall identity to human PGR protein.

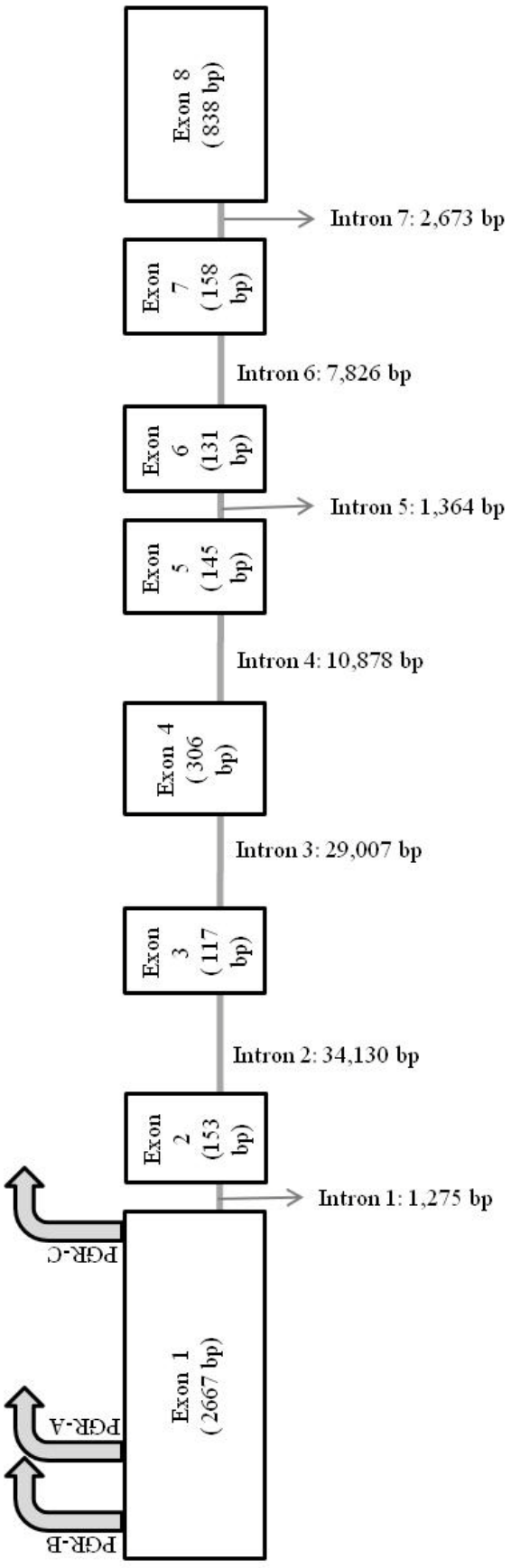
The porcine PGR protein was 75% identical to human PGR protein within the A/B (variable) domain and was 97% identical to human PGR protein within the C, D, and E domains. Homology in mouse and cow was also increased within the C, D, and E domain over the variable domain. Polymorphic sites within the sample population and between generated mRNA and genomic sequences were identified and resultant effects on protein sequence inferred.

INTRODUCTION

PGR is a member of the steroid nuclear receptor superfamily, along with estrogen receptor (ER) and androgen receptor (AR). Family members share a common protein structure with four distinct domains: an N-terminal variable domain (A/B), a DNA binding domain (C), hinge region containing the dimerization domain (D), and a hormone-binding domain (E) at the C terminus (DeGroot and Jameson, 2001). As expected, the variable region is the least conserved region among superfamily members.

In humans, three PGR mRNA isoforms (PGR-A, PGR-B, and PGR-C) arise from distinct, alternative promoters (Figure 3.1). The mRNA transcripts encode identical proteins, differing only in N-terminus truncations that may be regulated independently of one another and confer distinct biological functions (Mulac-Jericevic et al., 2000, 2004). The PGR isoforms have not yet been well characterized in pigs at either the mRNA or protein level. In pigs, the PGR gene is located on SSA 9p13-11 (Hu et al., 1998). The gene is nearly 100 kb in length and contains 8 exons. The presence or absence of specific

Figure 3.1 Representation of porcine PGR gene structure including exons and introns including sizes in base pairs (bp). The gene is located on SSC 9p13-11 (NCBI build 1.1; CU640401.2) with three distinct transcription initiation sites represented by arrows, giving rise to PGR-B, PGR-A, and PGR-C transcript isoforms, respectively (NCBI, Bethesda, MD).


Exon 8 (838 bp)
Intron 7: 2,673 bp
Exon 7 (158 bp)
Intron 6: 7,826 bp
Exon 6 (131 bp)
Intron 5: 1,364 bp
Exon 5 (145 bp)
Intron 4: 10,878 bp
Exon 4 (306 bp)
Intron 3: 29,007 bp
Exon 3 (117 bp)
Intron 2: 34,130 bp
Exon 2 (153 bp)
Intron 1: 1,275 bp
Exon 1 (2667 bp)
PGR-C
PGR-A
PGR-B

PGR isoforms has not yet been investigated in pig and neither the annotated PGR nucleotide nor amino acid sequences had been deposited in public databases when this project was undertaken. A comparison of identity between porcine PGR nucleotide and amino acid sequences and the sequences of better-characterized model species like human (*Homo sapiens*), mouse (*Mus musculus*) and cow (*Bos taurus*) may begin to elucidate the nature and function of PGR in the pig.

MATERIALS AND METHODS

Samples

Uteri from d 0 cyclic gilts were collected (n = 2) at Oklahoma State University (Ross et al., 2010). Gilts were hysterectomized, uterine horns dissected and endometrium manually separated from myometrium with sterile scissors. Endometrium was then flash frozen in liquid nitrogen. At the University of Missouri – Columbia, uterine tissue was collected from non-pregnant gilts by terminal hysterectomy (n = 2) at estrus (d 0). Endometrial tissue was manually isolated from myometrium, flash frozen in liquid nitrogen and stored at -80°C.

RNA isolation

Total RNA was isolated from approximately 500 mg of endometrium using RNAwiz (Ambion, Austin, TX) in accordance with the manufacturer's protocol at Oklahoma State University and resuspended in ddH_2O (Ross et al., 2010). Samples were transported to the University of Missouri – Columbia on dry ice and stored at -80°C.

Frozen endometrial samples collected at the University of Missouri – Columbia were ground into powder in liquid nitrogen. Total RNA was isolated from approximately 50 mg of endometrium using the Trizol Reagent protocol (Invitrogen, Carlsbad, CA).

University of Missouri isolated total RNA and Oklahoma State University total RNA samples were quantified by spectrophotometry on the NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE). Sample integrity was assessed by both 260:280 nm absorbance ratio and visualization of 28S and 18S ribosomal RNA on an 0.8% agarose gel (with 0.09 M Tris-borate, 0.002 M EDTA buffer and 0.5 µg/mL ethidium bromide). An aliquot of 5 µg of each total RNA sample (n = 4, 2 from OSU, 2 from MU) was treated with DNAse I enzyme (Ambion Inc) and incubated for an hour at 37°C. Efficacy of the DNAse treatment was confirmed visually on a 0.8% agarose gel.

cDNA synthesis

Five µg of total RNA and DNAse treated total RNA from each sample was used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random hexamer priming in a 100 µL reaction in accordance with manufacturer's protocol. Quantity yield of cDNA was assessed by spectrophotometry and quality was assessed both by 260:280 nm absorbance ratio

(NanoDrop-1000, NanoDrop Technologies, Wilmington, DE) and visualized on a 1.2% agarose gel.

Primer design

Human PGR mRNA sequence (NM000926.4) was aligned with porcine unannotated genomic contig (CU640401.2) using NCBI align2 sequences BLAST program to map PGR human exon location to the porcine genomic sequence (NCBI Bethesda, MD). The porcine genomic nucleotide sequence in predicted exonic regions was then used to design oligonucleotide primers using publicly available Primer3 software v 0.4.0 (Primer3, Totowa, NJ). Eight overlapping primer pairs spanning PGR from the 5' promoter region to the 3' UTR were developed (Table 3.1) for amplification and sequencing of the porcine PGR transcript.

Polymerase chain reaction (PCR)

Amplification using 7 primer pairs (Table 3.1, excluding PR_Ex1_GAP_F1, R1) was performed using Platinum® *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA). The Takara *LA Taq*™ DNA Polymerase with GC Buffers (TaKaRa Bio USA, Madison, WI) was used for one primer (PR_Ex1_GAP_F1, PR_Ex1_GAP_R1) pair to amplify a region enriched (69.5%) for guanine and cytosine residues (Table 3.1). A standard thermal cycler protocol of 35 cycles consisting of 94°C melting temperature for 15 seconds, 58°C annealing temperature for 20 seconds and 72°C extension temperature of 1 minute for products of expected size less than or equal to 1 kb (PR_UP_F6, R6; PR_5'_GAP_F2, R2; PR_B_F1, R1; PR_AB_F1, R2; PR_Ex1_GAP_F1, R1; PR_Ex8_GAP_F1, R2) and

2 minutes for products of expected size greater than 1 kb (PR_B_F3, PR_AB_R1; PR_AB_Ex1_F2, PR_C_Ex8_R2) with an initial denaturation and enzyme activation of 75 seconds at 94°C and a terminal 10 minute extension cycle at 65°C was used for both DNA polymerase enzymes. Reactions using both polymerases were prepared in 50 µl reactions with 0.2 µM each of forward and reverse primers and 100 ng template cDNA in accordance with manufacturer's protocols.

cDNA sequencing

Reactions of 16 µL PCR products (50 ng) with either the forward or reverse PCR primer (20 pmol) were prepared for sequencing on a 3730 96-capillary DNA Analyzer using the Big Dye Terminator (Applied Biosystems, Foster City, CA) method at the University of Missouri – Columbia DNA Core Facility. The product amplified using PR_Ex1_GAP_F1 and PR_Ex1_GAP_R1 primers was GC-rich and required a final concentration of 10% DMSO in the sequencing reaction due to the high content of guanine and cytosine residues (69.5%) in the sequence. No other primer pair (Table 3.1) required DMSO inclusion to relax secondary cDNA structure for successful sequencing.

Sequence analysis

Chromatographs generated by the ABI Sequence Analyzer software were visually assessed on the DNA Core Facility Laboratory Information Management System (LIMS) platform for polymorphic loci or additional unambiguous base detection. The sequences from overlapping primer sets spanning the porcine PGR coding sequence were assembled into a consensus contig using the publicly-available software CodonCode Aligner

(CodonCode Corporation, Dedham, MD) with the default settings. Identification of the sequence generated was validated using the NCBI BLAST program against human, mouse and bovine genomic builds. In all cases the highest BLAST score obtained was to annotated PGR sequence (NM000926.4, NM008829.2, XM583951.5; NCBI, Bethesda, MD).

Alignments of two sequences were performed by using the BLASTN and BLASTP functions of NCBI for nucleotide (mRNA and genomic) and protein sequences, respectively (NCBI, Bethesda, MD). The BLAST program uses a *k-tuple* or word method of alignments in which non-overlapping subsets of length *k* in the sequence are compared for identity. The default setting is $k = 28$ for BLASTN and $k = 3$ for BLASTP (NCBI, Bethesda, MD). Identity percentages generated by this function were reported. The method calculates pairwise comparisons of identity between each pair of sequences using the equation:

Reported homology = (# identities in alignment / # residues compared)

Gaps are excluded from the calculation.

Inferred protein sequence was predicted from porcine mRNA sequence (GQ903679.1) using publicly-available NCBI ORF Finder software (NCBI, Bethesda, MD). Inferred protein sequence was also generated from the genomic sequence (CU640401.2) derived putative exons. These sequences were compared using the BLASTP program. The mRNA predicted protein was also aligned to human, mouse and predicted bovine protein sequences (NP000917.3, NP032855.2, and XP583951.5, respectively) using the BLASTP function in NCBI(NCBI, Bethesda, MD).

Publicly-available ClustalW2 v 2.0.11 software from European Bioinformatics Institute (EBI, Hinxton, Cambridge, UK) was used on default settings for multiple sequence alignment of pig, cow, mouse and human PGR mRNA (GQ903679.1, XM583951.5, NM008829.2 and NM000926.4, respectively) and protein (NP001159960.1, XP583951.5, NP032855.2 and NP000917.3, respectively) sequences. ClustalW2 v 2.0.11 uses a hierarchical method of alignment in which the sequences sharing the greatest identity are aligned first, then less similar sequences are added. The method calculates pairwise comparisons of identity between each pair of sequences using the equation:

Reported homology = (# identities in optimal alignment / # residues compared)

Gaps are excluded from the calculation (EBI, Hinxton, Cambridge, UK).

RESULTS

Porcine PGR mRNA was sequenced from 8 cDNA PCR products and assembled to form a full-length transcript of 4.3 kb (Figure 3.1; Table 3.1). Using DNA to amplify the promoter region and cDNA to amplify the mature mRNA transcript, a full-length nucleotide sequence for porcine PGR mRNA was built (Figure 3.2). The mRNA sequence generated a BLAST alignment with high significance ($P < 0.001$) to an unannotated genomic contig located on SSA 9 (CU640401.2; Figure 3.3).

Table 3.1 Primer names, sequences and locations used for amplification and sequencing porcine PGR. Location indicates position in GenBank GQ903679.1; exon location is noted. Primer pair PR_UP_F6, R6 is located in the promoter region of the gene and was amplified in genomic DNA template. The remaining primer pairs amplified products from cDNA template. Primer pairs PR_5'_GAP_F2, R2 and PR_B_F1, R1 amplified the 5' UTR of the PGR-B transcript. Primer pair PR_B_F3, PR_AB_R1 spans the ATG translation start codons of PGR-B and PGR-A. Primer pair PR_AB_F1, R2 amplified a 3' region of exon 1 common to both PGR-B and PGR-A. Primer pairs PR_Ex1_GAP_F1, R1 and PR_AB_Ex1_F2, PR_C_Ex8_R2 span exons 1 and 2 and exons 2 through 8, respectively. Primer pair PR_Ex8_GAP_F1, R2 is located in the 3' UTR of exon 8. GenBank sequence GQ903679.1 ends at base 4515 although PR_Ex8_GAP_R2 extended to 4518 because the final 3 bases could not be validated on a chromatograph.

Primer Name	Sequence	Exon (Base Location)
PR_UP_F6	TCT TCT TCC AGG GAA AAA TCT	Exon 1(1 – 21)
PR_UP_R6	TAG CTG GCG ACA GTC ATC TC	Exon 1 (451 – 470)
PR_5'_GAP_F2	ATC TGC ACC TAC AGG CGT TT	Exon 1 (394 – 413)
PR_5'_GAP_R2	GGG ATC TCG TTT CCT AAT GG	Exon 1 (729 – 749)
PR_B_F1	TCA GAC TGA AGT CGG GGA AC	Exon 1 (692 – 711)

Primer Name	Sequence	Exon (Base Location)
PR_B_R1	GGG TGA AAT CTC CAC CTC CT	Exon 1 (870 – 889)
PR_B_F3	GAC CCA GGA GGT GGA GAT TT	Exon 1 (865 – 884)
PR_AB_R1	GAT GGG CAC GTG GAT AAA AT	Exon 1 (1927 – 1946)
PR_AB_F1	GCT CCA TGG TTC CAC TTC TG	Exon 1 (1714 – 1733)
PR_AB_R2	GCC TTC CTC CTC TTC CTT GA	Exon 1 (2182 – 2201)
PR_Ex1_GAP_F1	GCC CTG AAG ATC AAG GAA GA	Exon 1 (2172 – 2191)
PR_Ex1_GAP_R1	GAT GCT TCA TCC CCA CAG AT	Exon 2 (2736 – 2755)
PR_AB_Ex1_F2	GCA GGT GTA CCA GCC CTA TC	Exon 1 (2636 – 2655)
PR_C_Ex8_R2	GAG AAG GGG TTT CAC CAT CC	Exon 8 (3799 – 3818)
PR_Ex8_GAP_F1	TGC TGC ACA ATT ACC CAA GA	Exon 8 (3770 – 3789)
PR_Ex8_GAP_R2	CAG TGA AAA CCT ACA AAA CCC ATA	Exon 8 (4495 – 4518)

Figure 3.2 Nucleotide sequence of the full-length porcine PGR mRNA transcript including additional sequence 5' to the putative PGR-B transcription initiation site. Base numbers are shown. Nucleotides are abbreviated: a = adenine, t = thymidine, c = cytosine, g = guanine. Sequences were generated from pig endometrium tissues using DNA (putative promoter region) and cDNA (transcribed region) templates. GenBank accession number assigned to the generated sequence was GQ903679.1.

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   1 ttcttcttcc agggaaaaat ctttacaaaa ttgaaatctt gaaaattcag cttctgatag
  61 aatccattcg cttttccagt tagactgggc ataattctta attggtagta cacaatctaa
 121 tatgcttacg aagttccatc ctcacagtac ctgtttttta gactagcggt tgggaatact
 181 aactccagaa tttcagatct ggtccataat tggggcaggg aggggctttg ggcggagcct
 241 ccggataggg aggaggcatt gttagaaagc tgtggggcta gtctactggc tgtcactact
 301 ctagttaagc ctttgtattt gaatgtgcga ggagggtctg ctgtgttagc agcattttca
 361 gtgagaacta gtctcacttg gcatttgagt ggaatctgca cctacaggcg tttcgtcccc
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 481 ggaggtataa gactgttcag ggaatgggct gtgccgagag gccaaaatag ccccagggtt
 541 ttagggagac gggaaatgta acacaggcag ggactggcag caacagggca ttgaagccgg
 601 agaaaaagtc gggagataga gccgccagta attgctttcg aagccgccgc cactcgagtg
 661 ctgacttgtg agtactctgc gtctcctgtc ttcagactga agtcggggaa ctctcttgga
 721 gaactctccc agttaggaaa cgagatccct acaactacct tcgccggctc tccccacctg
 781 cctcaaggac gtaacaacta tcacccatct cctgcctacc agacgctggg caagggcagg
 841 agctgacagg tgccgccccc ttccgaccca ggaggtggag atttcacccc cctctccacc
 901 gtcccgtcca gccaaattca acacctattt tctcctcccg ctgcccctat attcccgaca
 961 ccccctcatc cttcccctat ccctcctccc cagagacagg ggaggagaaa aggggagtcc
1021 aggtcgtcat gactgagctg aaggcaaagg atccccgggc tccccacgtg gcgggcagag
1081 cgccctcccc cacccagctc gggacgctgg gacgcccaga cacaggcccc tttcaggcga
1141 gccagacctc ggaagcgtcg cccgcagcct cggccatacc cctctccctg gacgggctac
1201 tcttccctgg gccctgccag ggacaggaac cagacgggaa gacgcaggac cagcagtcgc
1261 tgtcagacgt ggaggggggcg tatcccacag ttgaagctac agagggtgct ggaggtggca
1321 gctctagacc ctcggaaaaa gacaccgggc tgctggacag tgtcttggac acgctactag
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1561 ccgcccataa agtgctatcc aggggtctgg caccgtcccg gcagctgctg ccctcgactg
1621 ccgggagcca tcactggccg gcggccgcag tgaagccctc tccgcagccc gccgtggtgg
1681 aggtggagga ggaggatgac tccgagtccg agggctccat ggttccactt ctgaagggta
1741 aaccccggcc tgcaggaggc acggctgccg gaacaggaac cccgaccggt gctcctggga
1801 cggccgcagg aggcgtcgcc ctggtcccca aggaagatgc ccgcttctcg gcgcccaggg
1861 gcgccctggc ggagcacgat gcgtcagggg cgcccgggcg ctcccctctg gccactacgg
1921 tgatggattt tatccacgtg cccatcctgc cgctcaacac ggcctttctg gccgcccgca
1981 cccggcaact gctagagggg gataactatg acggcggggc cccggctgtc agcgcctttg
2041 caccgccgcg gggctcgccc tcggccccgt ccgcctccgt caccgccggc gacttccccg
2101 actgcgcgta ctctgcagac gacgagccca aggacgacgc attcccgctc tatggcgact
2161 tccagccgcc cgccctgaag atcaaggaag aggaggaagg cgccgaggcc gccgcgcgct
2221 ctccgcggtc gtacctggtg gcgggtgcca accctgccgt cttcccggat tttccgctgg
2281 cgccgccgtc gctgcctccg cgagcgtcgt ccagacctgg ggaagcctcg gcggcggtcg
2341 cacccaccag tgcctcggta tcgtcggcgt cctcgtcggg gtcggccttg gagtgcatgt
2401 tgtataaggc ggagggcgcg ccgccccaac agggcccgtt cccgcctccg ccctgcaagc
2461 cgccgagcgc cggcgcctgc ctgctgccgc gggacagcct gccgtccacc tcggcctccg
2521 ccgccgccgc cgccgccgcc gccgccgggg cggctcctgc gctctaccag ccgcttggcc
2581 ttaacgggct cccgcagctc ggctaccagg ccgcggtact caaggagggc ctgccgcagg
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2761 gtcattatgg tgtccttacc tgtgggagct gtaaggtctt ctttaaaagg gcaatggaag
2821 ggcagcacaa ctacttatgt gctggaagaa acgactgcat tgttgataaa atccgcagaa
2881 aaaactgccc agcttgtcgc cttagaaagt gctgtcaggc tggcatggtc cttggaggtc
2941 gaaagtttaa aaagttcaat aaagttagag ttatgagagc actagatgcc gttgctctcc
3001 cacagtcggg gggcattcca aatgaaagtc aggccctaag ccagagaatc acttttcac
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3361 attttgcacc tgatctaata ctaaatgaac agcggatgaa ggaatcatca ttctattcat
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3421 tatgccttac catgtggcag atcccacagg agtttgtgaa gcttcaagtt agccaagaag
3481 agtttctctg tatgaaagta ttgctacttc ttaatacaat tcctttggaa ggactaagaa
3541 gtcaaaacca gtttgaagag atgagatcaa gctacattag agagctcatc aaggcaattg
3601 gtttgagaca aaaaggagtc gtccctagct cacagcgttt ctaccagctt acaaaacttc
3661 ttgataactt gcatgatctt gtcaaacaac ttcatctgta ctgcttgaat acatttatcc
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3781 tacccaagat cttggcaggg atggtgaaac cccttctctt tcataaaaag tgaatgccat
3841 ttttatcttt aaaaaattaa actttgtggt atatctgttt tcgtttttgt caggattatg
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3961 aatttcagag gaaaaatgtg agggtgaatt ggatttgctt tataaagcat tatttgaatt
4021 tttagtgttt ttgcgtaccc atattttctt taagagttta cacgattgaa aaagtactaa
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4201 agctattata tatgttcctt ctatgtagct ccctttgtct ccctgattat atttacaaaa
4261 ctgaaacttt aaaatggtat gcaaaacttg tctatatgag tgtacataca ttttttttgtg
4321 agaaggatat ttataatctt aatcagtttt tccaaagggt ttttaatgca aaaatatata
4381 tatacagaaa aagagttaac aattctttaa ttagatgata ctcctcaaac taggaaaacc
4441 agcttatatg ttaagactgt ttcccagatt ggaaacacaa atctctaggg aagttattaa
4501 gtagagccat agcag

Figure 3.3. Alignment of porcine PGR mRNA (GQ903679.1) and genomic (CU640401.2) sequences. Alignment generated by BLASTN program (NCBI). Query (top) denotes mRNA sequence; Sbjct (bottom) denotes genomic sequence and locations (bases) in the sequence are shown. Nucleic acid residues are listed in FASTA format (A = adenine; T = thymidine; C = cytosine; G = guanine; dashes = gaps; vertical lines = identities). Sequence represented in lowercase, gray letters indicate regions of low sequence complexity. The number of discrepancies between the two sequences is 15 out of 4514 residues (0.003%). The first mRNA residue did not have sufficient power to align to genomic sequence. Of the mismatches, 4 are gaps in the sequence (0.0009%). Exons are labeled. The translation start codons (ATG) are shown in bold, underlined text: PGR-B at base 1029 (reference sequence is P: GQ903679.1); PGR-A at base 1516; PGR-C at base 2815. The stop codon (TGA) located at base 3829 is shown in italics, double underlined text.

PROMOTER AND EXON 1

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Query  2      TCTTCTTCCAGGGAAAAATCTTTACAAAATTGAAATCTTGAAAATTCAGCTTCTGATAGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28712  TCTTCTTCCAGGGAAAAATCTTTACAAAATTGAAATCTTGAAAATTCAGCTTCTGATAGA

Query  62     ATCCATTCGCTTTTCCAGTTAGACTGGGCATAATTCTTAATTGGTAGTACACAATCTAAT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28772  ATCCATTCGCTTTTCCAGTTAGACTGGGCATAATTCTTAATTGGTAGTACACAATCTAAT

Query  122    ATGCTTACGAAGTTCCATCCTCACAGTACCTGTTTTTTAGACTAGCGGTTGGGAATACTA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28832  ATGCTTACGAAGTTCCATCCTCACAGTACCTGTTTTTTAGACTAGCGGTTGGGAATACTA

Query  182    ACTCCAGAATTTCAGATCTGGTCCATAATTGGGGCAGGGAGGGGCTTTGGGCGGAGCCTC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28892  ACTCCAGAATTTCAGATCTGGTCCATAATTGGGGCAGGGAGGGGCTTTGGGCGGAGCCTC

Query  242    CGGATAGGGAGGAGGCATTGTTAGAAAGCTGTGGGGCTAGTCTACTGGCTGTCACTACTC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28952  CGGATAGGGAGGAGGCATTGTTAGAAAGCTGTGGGGCTAGTCTACTGGCTGTCACTACTC

Query  302    TAGTTAAGCCTTTGTATTTGAATGTGCGAGGAGGGTCTGCTGTGTTAGCAGCATTTTCAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29012  TAGTTAAGCCTTTGTATTTGAATGTGCGAGGAGGGTCTGCTGTGTTAGCAGCATTTTCAG

Query  362    TGAGAACTAGTCTCACTTGGCATTTGAGTGGAATCTGCACCTACAGGCGTTTCGTCCCCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29072  TGAGAACTAGTCTCACTTGGCATTTGAGTGGAATCTGCACCTACAGGCGTTTCGTCCCCT

Query  422    CCGGACTGCTAAGAAATGTGAGGGCTGCGGAGATGACTGTCGCCAGCTATAGGGAACCGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29132  CCGGACTGCTAAGAAATGTGAGGGCTGCGGAGATGACTGTCGCCAGCTATAGGGAACCGG

Query  482    GAGGTATAAGACTGTTCAGGGAATGGGCTGTGCCGAGAGGCCAAAATAGCCCCAGGGTTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29192  GAGGTATAAGACTGTTCAGGGAATGGGCTGTGCCGAGAGGCCAAAATAGCCCCAGGGTTT

Query  542    TAGGGAGACGGGAAATGTAACACAGGCAGGGACTGGCAGCAACAGGGCATTGAAGCCGGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29252  TAGGGAGACGGGAAATGTAACACAGGCAGGGACTGGCAGCAACAGGGCATTGAAGCCGGA

Query  602    GAAAAAGTCGGGAGATAGAGCCGCCAGTAATTGCTTTCGAAGCCGCCGCCACTCGAGTGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29312  GAAAAAGTCGGGAGATAGAGCCGCCAGTAATTGCTTTCGAAGCCGCCGCCACTCGAGTGC

Query  662    TGACTTGTGAGTACTCTGCGTCTCCTGTCTTCAGACTGAAGTCGGGGAACTCTCTTGGAG
              |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  29372  TGACTTGTGAGTACTCTGCGTCTCCTGTCCTCAGACTGAAGTCGGGGAACTCTCTTGGAG

Query  722    AACTCTCCCAGTTAGGAAACGAGATCCCTACAACTACCTTCGCCGGCTCTCCCCACCTGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29432  AACTCTCCCAGTTAGGAAACGAGATCCCTACAACTACCTTCGCCGGCTCTCCCCACCTGC

Query  782    CTCAAGGACGTAACAACTATCACCCATCTCCTGCCTACCAGACGCTGGGCAAGGGCAGGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29492  CTCAAGGACGTAACAACTATCACCCATCTCCTGCCTACCAGACGCTGGGCAAGGGCAGGA
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Query  842    GCTGACAGGTGCCGCCCCCTTCCGACCCAGGAGGTGGAGATTTCACCCCCCTCTCCACCG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29552  GCTGACAGGTGCCGCCCCCTTCCGACCCAGGAGGTGGAGATTTCACCCCCCTCTCCACCG

Query  902    TCCCGTCCAGCCAAATTCAACACCTATTTTctcctcccgctgcccctatattcccgacac
              |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  29612  TCCCGTCCAGCCAAATTCAACACCTATTTTCTCCTCCCGCTGCCCCTATATTTCCGACAC

Query  962    cccctcatccttccccctatccctcctccccAGAGACAGGGGAGGAGAAAAGGGGAGTCCA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29672  CCCCTCATCCTTCCCCTATCCCTCCTCCCCAGAGACAGGGGAGGAGAAAAGGGGAGTCCA

Query  1022   GGTCGTCATGACTGAGCTGAAGGCAAAGGATCCCCGGGCTCCCCACGTGGCGGGCAGAGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29732  GGTCGTCATGACTGAGCTGAAGGCAAAGGATCCCCGGGCTCCCCACGTGGCGGGCAGAGC

Query  1082   GCCCTCCCCCACCCAGCTCGGGACGCTGGGACGCCCAGACACAGGCCCCTTTCAGGCGAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29792  GCCCTCCCCCACCCAGCTCGGGACGCTGGGACGCCCAGACACAGGCCCCTTTCAGGCGAG

Query  1142   CCAGACCTCGGAAGCGTCGCCCGCAGCCTCGGCCATACCCCTCTCCCTGGACGGGCTACT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29852  CCAGACCTCGGAAGCGTCGCCCGCAGCCTCGGCCATACCCCTCTCCCTGGACGGGCTACT

Query  1202   CTTCCCTGGGCCCTGCCAGGGACAGGAACCAGACGGGAAGACGCAGGACCAGCAGTCGCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29912  CTTCCCTGGGCCCTGCCAGGGACAGGAACCAGACGGGAAGACGCAGGACCAGCAGTCGCT

Query  1262   GTCAGACGTGGAGGGGGCGTATCCCACAGTTGAAGCTACAGAGGGTGCTGGAGGTGGCAG
              |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  29972  GTCAGACGTGGAGGGGGCGTATCCCAGAGTTGAAGCTACAGAGGGTGCTGGAGGTGGCAG

Query  1322   CTCTAGACCCTCGGAAAAAAGACACCGGGCTGCTGGACAGTGTCTTGGACACGCTACTAGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30032  CTCTAGACCCTCGGAAAAAAGACACCGGGCTGCTGGACAGTGTCTTGGACACGCTACTAGC

Query  1382   GCCCTCAGGTCCCGGGCAGAGCCACGCCAGCCCTCCCGCCTGCGAAGCCACCAGCCCTTG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30092  GCCCTCAGGTCCCGGGCAGAGCCACGCCAGCCCTCCCGCCTGCGAAGCCACCAGCCCTTG

Query  1442   GTGCTTGTTTGGCTCTGAGCTTCCCCAGGACGCTCGGGTTGCCCCTTCCACCCAGGGAGT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30152  GTGCTTGTTTGGCTCTGAGCTTCCCCAGGACGCTCGGGTTGCCCCTTCCACCCAGGGAGT

Query  1502   ATTGCCCCTGCTCATGAGCCGGCCAGAGGGCAAGGCTGGCGACAGCTCCGGGACGGCAGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30212  ATTGCCCCTGCTCATGAGCCGGCCAGAGGGCAAGGCTGGCGACAGCTCCGGGACGGCAGC

Query  1562   CGCCCATAAAGTGCTATCCAGGGGTCTGGCACCGTCCCGGCAGCTGCTGCCCTCGACTGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30272  CGCCCATAAAGTGCTATCCAGGGGTCTGGCACCGTCCCGGCAGCTGCTGCCCTCGACTGC

Query  1622   CGGGAGCCATCACTGGCCGGCGGCCGCAGTGAAGCCCTCTCCGCAGCCCGCCGTGGTGGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30332  CGGGAGCCATCACTGGCCGGCGGCCGCAGTGAAGCCCTCTCCGCAGCCCGCCGTGGTGGA

Query  1682   GGTGGAGGAGGAGGATGACTCCGAGTCCGAGGGCTCCATGGTTCCACTTCTGAAGGGTAA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30392  GGTGGAGGAGGAGGATGACTCCGAGTCCGAGGGCTCCATGGTTCCACTTCTGAAGGGTAA
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Query  1742   ACCCCGGCCTGCAGGAGGCACGGCTGCCGGAACAGGAACCCCGACCGGTGCTCCTGGGAC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30452  ACCCCGGCCTGCAGGAGGCACGGCTGCCGGAACAGGAACCCCGACCGGTGCTCCTGGGAC

Query  1802   GGCCGCAGGAGGCGTCGCCCTGGTCCCCAAGGAAGATGCCCGCTTCTCGGCGCCCAGGGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30512  GGCCGCAGGAGGCGTCGCCCTGGTCCCCAAGGAAGATGCCCGCTTCTCGGCGCCCAGGGG

Query  1862   CGCCCTGGCGGAGCACGATGCGTCAGGGGCGCCCGGGCGCTCCCCTCTGGCCACTACGGT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30572  CGCCCTGGCGGAGCACGATGCGTCAGGGGCGCCCGGGCGCTCCCCTCTGGCCACTACGGT

Query  1922   GATGGATTTTATCCACGTGCCCATCCTGCCGCTCAACACGGCCTTTCTGGCCGCCCGCAC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30632  GATGGATTTTATCCACGTGCCCATCCTGCCGCTCAACACGGCCTTTCTGGCCGCCCGCAC

Query  1982   CCGGCAACTGCTAGAGGGGGATAACTATGACGGCGGGGCCCCGGCTGTCAGCGCCTTTGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30692  CCGGCAACTGCTAGAGGGGGATAACTATGACGGCGGGGCCCCGGCTGTCAGCGCCTTTGC

Query  2042   ACCGCCGCGGGGCTCGCCCTCGGCCCCGTCCGCCTCCGTCACCGCCGGCGACTTCCCCGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30752  ACCGCCGCGGGGCTCGCCCTCGGCCCCGTCCGCCTCCGTCACCGCCGGCGACTTCCCCGA

Query  2102   CTGCGCGTACTCTGCAGACGACGAGCCCAAGGACGACGCATTCCCGCTCTATGGCGACTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30812  CTGCGCGTACTCTGCAGACGACGAGCCCAAGGACGACGCATTCCCGCTCTATGGCGACTT

Query  2162   CCAGCCGCCCGCCCTGAAGATCAAGGAAGAGGAGGAAGGCGCCGAGGCCGCCGCGCGCTC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30872  CCAGCCGCCCGCCCTGAAGATCAAGGAAGAGGAGGAAGGCGCCGAGGCCGCCGCGCGCTC

Query  2222   TCCGCGGTCGTACCTGGTGGCGGGTGCCAACCCTGCCGTCTTCCCGGATTTTCCGCTGGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30932  TCCGCGGTCGTACCTGGTGGCGGGTGCCAACCCTGCCGTCTTCCCGGATTTTCCGCTGGC

Query  2282   GCCGCCGTCGCTGCCTCCGCGAGCGTCGTCCAGACCTGGGGAAGCCTCGGCGGCGGTCGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  30992  GCCGCCGTCGCTGCCTCCGCGAGCGTCGTCCAGACCTGGGGAAGCCTCGGCGGCGGTCGC

Query  2342   ACCCACCAGTGCCTCGGTATCGTCGGCGTCCTCGTCGGGGTCGGCCTTGGAGTGCATGTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31052  ACCCACCAGTGCCTCGGTATCGTCGGCGTCCTCGTCGGGGTCGGCCTTGGAGTGCATGT-

Query  2402   GTATAAGGCGGAGGGCGCGCCGCCCCAACAGGGCCCGTTCCCGCCTCCGCCCTGCAAGCC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31111  GTATAAGGCGGAGGGCGCGCCGCCCCAACAGGGCCCGTTCCCGCCTCCGCCCTGCAAGCC

Query  2462   GCCGAGcgccggcgcctgcctgctgccgcgggacagcctgccgtccacctcggcctccgc
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31171  GCCGAGCGCCGGCGCCTGCCTGCTGCCGCGGGACAGCCTGCCGTCCACCTCGGCCTCCGC

Query  2522   cgccgccgccgccgccgccgccgccgGGGCGGCTCCTGCGCTCTACCAGCCGCTTGGCCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31231  CGCCGCCGCCGCCGCCGCCGCCGGGGCGGCTCCTGCGCTCTACCAGCCGCTTGGCCT

Query  2582   TAACGGGCTCCCGCAGCTCGGCTACCAGGCCGCGGTACTCAAGGAGGGCCTGCCGCAGGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  31291  CAACGGGCTCCCGCAGCTCGGCTACCAGGCCGCGGTACTCAAGGAGGGCCTGCCGCAGGT
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Query  2642   GTACCAGCCCTATCTCAACTACCTGAGG
              ||||||||||||||||||||||||||||
Sbjct  31351  GTACCAGCCCTATCTCAACTACCTGAGG
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EXON 2

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Query  2667   AGGCCGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTCGAGTCATTACCTCAGAAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32613  AGGCCGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTCGAGTCATTACCTCAGAAG

Query  2727   ATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  32673  ATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGG

Query  2787   AGCTGTAAGGTCTT-CTTTAAAAGGGCA<u>**ATG**</u>GAAGG
              |||||||||||||| ||| ||||||||||||||||
Sbjct  32733  AGCTGTAAGGTCTTTCTT-AAAAGGGCA<u>**ATG**</u>GAAGG
```

EXON 3

```
Query  2819   AGGGCAGCACAACTACTTATGTGCTGGAAGAAACGACTGCATTGTTGATAAAATCCGCAG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65332  AGGGCAGCACAACTACTTATGTGCTGGAAGAAACGACTGCATTGTTGATAAAATCCGCAG

Query  2879   AAAAAACTGCCCAGCTTGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTCCTTGGAGG
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65392  AAAAAACTGCCCAGCTTGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTCCTTGGAGG

Query  2939   T
              |
Sbjct  65452  T
```

EXON 4

```
Query  2936   AGGTCGAAAGTTTAAAAAGTTCAATAAAGTTAGAGTTATGAGAGCACTAGATGCCGTTGC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94317  AGGTCGAAAGTTTAAAAAGTTCAATAAAGTTAGAGTTATGAGAGCACTAGATGCCGTTGC

Query  2996   TCTCCCACAGTCGGGGGGCATTCCAAATGAAAGTCAGGCCCTAAGCCAGAGAATCACTTT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94377  TCTCCCACAGTCGGGGGGCATTCCAAATGAAAGTCAGGCCCTAAGCCAGAGAATCACTTT

Query  3056   TTCACCAAATCAAGACTTACAGCTGATCCCCCCGTTGATCAACTTGCTCATGAGCATTGA
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94437  TTCACCAAATCAAGACTTACAGCTGATCCCCCCGTTGATCAACTTGCTCATGAGCATTGA

Query  3116   ACCAGATGTGGTCTATGCAGGACATGACAACACCAAACCCGACACTTCGAGTTCTTTGCT
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94497  ACCAGATGTGGTCTATGCAGGACATGACAACACCAAACCCGACACTTCGAGTTCTTTGCT

Query  3176   GACAAGTCTGAATCAGCTGGGTGAAAGACAACTTCTTTCAGTAGTCAAGTGGTCTAAGTC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  94557  GACAAGTCTGAATCAGCTGGGTGAAAGACAACTTCTTTCAGTAGTCAAGTGGTCTAAGTC
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Query  3236   ACTGCCAGGT
              ||||||||||
Sbjct  94617  ACTGCCAGGT
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EXON 5

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Query  3242    AGGTTTTCGAAACTTACATATTGATGACCAAATAACTCTCATCCAGTATTCCTGGATGAG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104927  AGGTTTTCGAAACTTACATATTGATGACCAAATAACTCTCATCCAGTATTCCTGGATGAG

Query  3302    CTTAATGGTATTTGGACTAGGATGGAGATCCTATAAACATGTCAGTGGACAGATGCTTTA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104987  CTTAATGGTATTTGGACTAGGATGGAGATCCTATAAACATGTCAGTGGACAGATGCTTTA

Query  3362    TTTTGCACCTGATCTAATACTAAATGA
               ||||||||||||||||||||||||||
Sbjct  105047  TTTTGCACCTGATCTAATACTAAATGA
```

EXON 6

```
Query  3389    ACAGCGGATGAAGGAATCATCATTCTATTCATTATGCCTTACCATGTGGCAGATCCCACA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  106672  ACAGCGGATGAAGGAATCATCATTCTATTCATTATGCCTTACCATGTGGCAGATCCCACA

Query  3449    GGAGTTTGTGAAGCTTCAAGTTAGCCAAGAAGAGTTTCTCTGTATGAAAGTATTGCTACT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  106732  GGAGTTTGTGAAGCTTCAAGTTAGCCAAGAAGAGTTTCTCTGTATGAAAGTATTGCTACT

Query  3509    TCTTAATACAA
               ||||||||||| 
Sbjct  106792  TCTTAATACAA
```

EXON 7

```
Query  3509    TCTTAATACAATTCCTTTGGAAGGACTAAGAAGTCAAAACCAGTTTGAAGAGATGAGATC
               |||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118494  TCTTCATACAGTTCCTTTGGAAGGACTAAGAAGTCAAAACCAGTTTGAAGAGATGAGATC

Query  3569    AAGCTACATTAGAGAGCTCATCAAGGCAATTGGTTTGAGACAAAAAGGAGTCGTCCCTAG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118554  AAGCTACATTAGAGAGCTCATCAAGGCAATTGGTTTGAGACAAAAAGGAGTCGTCCCTAG

Query  3629    CTCACAGCGTTTCTACCAGCTTACAAAACTTCTTGATAACTTGCATGAT
               ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  118614  CTCACAGCGTTTCTACCAGCTTACAAAACTTCTTGATAACTTGCATGAT
```

EXON 8

```
Query  3678    CTTGTCAAACAACTTCATCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121626  CTTGTCAAACAACTTCATCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGT
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Query  3738    GTTGAATTTCCAGAAATGATGTCTGAGGTTATTGCTGCACAATTACCCAAGATCTTGGCA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121686  GTTGAATTTCCAGAAATGATGTCTGAGGTTATTGCTGCACAATTACCCAAGATCTTGGCA

Query  3798    GGGATGGTGAAACCCCTTCTCTTTCATAAAAAGTGAATGCCATTTTTATCTTTAAAAAAT
               |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121746  GGGATGGTGAAACCTCTTCTCTTTCATAAAAAGTGAATGCCATTTTTATCTTTAAAAAAT

Query  3858    TAAACTTTGTGGTATATCTGTTTTCGTTTTTGTCAGGATTATGAGGTCTTGAGTTTTTAT
               |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  121806  TAAACTTTGTGGTATATCTGTTTTCGTTTTTGTCAGGGTTATGAGGTCTTGAGTTTTTAT

Query  3918    AATGTTCTCAAAGCCCTACATTTATAACATATTGTACTGTGTAAATTTCAGAGG-AAAAA
               |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  121866  AATGTTCTCAAAGCCCTACATTTATAACATATTGTACTGTGTAAATTTCAGAGGAAAAAA

Query  3977    TGTGAGGGTGAATTGGATTTGCTTTATAAAGCATTATTTGAATTTTTAGTGTTTTTGCGT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121926  TGTGAGGGTGAATTGGATTTGCTTTATAAAGCATTATTTGAATTTTTAGTGTTTTTGCGT

Query  4037    ACCCATATTTTCTTTAAGAGTTTACACGATTGAAAAAGTACTAAGTTGATTGTTAAAGTT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121986  ACCCATATTTTCTTTAAGAGTTTACACGATTGAAAAAGTACTAAGTTGATTGTTAAAGTT

Query  4097    AATCCTATCCGTATCATTTCATACCATTTAGGTGAGATTTTTAACTTTCACAGCTAACAG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122046  AATCCTATCCGTATCATTTCATACCATTTAGGTGAGATTTTTAACTTTCACAGCTAACAG

Query  4157    ATCCTCTACTTTAGAGGaaaaaaatcttacacataaaaataaaaaGCTATTATATATGTT
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122106  ATCCTCTACTTTAGAGGAAAAAAATCTTACACATAAAAATAAAAAGCTATTATATATGTT

Query  4217    CCTTCTATGTAGCTCCCTTTGTCTCCCTGATTATATTTACAAAACTGAAACTTTAAAATG
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122166  CCTTCTATGTAGCTCCCTTTGTCTCCCTGATTATATTTACAAAACTGAAACTTTAAAATG

Query  4277    GTATGCAAAACTTGTCTATATGAGTGTACATACAttttttGTGAGAAGGATATTTATAA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122226  GTATGCAAAACTTGTCTATATGAGTGTACATACATTTTTTTGTGAGAAGGATATTTATAA

Query  4337    TCTTAATCAGTTTTTCCAAAGGGTTTTTAATGCAAAAATATATATATACAGAAAAAGAGT
               |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  122286  TCTTAATCAGTTTTTCCGAAGGGTTTTTAATGCAAAAATATATATATACAGAAAAAGAGT

Query  4397    TAACAATTCTTTAATTAGATGATACTCCTCAAACTAGGAAAACCAGCTTATATGTTAAGA
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122346  TAACAATTCTTTAATTAGATGATACTCCTCAAACTAGGAAAACCAGCTTATATGTTAAGA

Query  4457    CTGTTTCCCAGATTGGAAACACAAATCTCTAGGGAAGTTATTAAGTAGAGCCATAGCA
               |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122406  CTGTTTCCCAGATGGGAAACACAAATCTCTAGGGAAGTTATTAAGTAGAGCCATAGCA
```

Porcine PGR mRNA (GQ903679.1) was aligned to bovine (XM583951.5), murine (NM00829.2) and human (NM000926.4) PGR transcripts using the BLASTN program (Figure 3.4). All sequences aligned to the pig PGR scaffold, with low sequence coverage in the 5'- and 3'- most regions. Comparison between pig and cow transcripts uncovered 2 gaps in which no nucleotides was observed in cow sequence to align with pig sequence (Figure 3.4). The gaps in sequence between cow and pig resulted in 3 distinct regions of homology. The observed identity in these regions was, from 5' to 3', 79% (1235/1550 nucleotides), 94% (723/767 nucleotides) and 93% (305/326 nucleotides), respectively with a frequency of gaps within the homologous regions of 0% (1/767 nucleotides), 7% (112/1550 nucleotides) and 3% (12/326 nucleotides), respectively (Figure 3.5).

Comparison between pig and cow transcripts uncovered 2 regional gaps in which no cow sequence existed to align with pig sequence (Figure 3.4). The gaps in sequence between cow and pig resulted in 3 distinct regions of homology. The observed identity in these regions was, from 5' to 3', 79% (1235/1550 nucleotides), 94% (723/767 nucleotides) and 93% (305/326 nucleotides), respectively with a frequency of gaps within the homologous regions of 0% (1/767 nucleotides), 7% (112/1550 nucleotides) and 3% (12/326 nucleotides), respectively (Figure 3.5). Both mouse and human PGR mRNA sequences aligned continuously with pig PGR. The observed identity between pig and mouse mRNA sequences was 2377/2989 nucleotides (79%) with 176 gaps (5%; Figure 3.6). The observed identity between pig and human mRNA sequences was 3588/4256 nucleotides (84%) with 221 gaps (5%; Figure 3.7).

Figure 3.4 Representation of nucleotide alignment between porcine PGR mRNA and bovine, murine and human PGR mRNA transcripts. Alignment generated by BLASTN program (NCBI). Thick top line represents 4515 nucleotide pig PGR mRNA (GQ903679.1). Thinner lines below represent, from top to bottom: 2688 nucleotide cow PGR mRNA (XM583951.5), 6889 nucleotide mouse PGR mRNA (NM008829.2) and 13,037 nucleotide human PGR mRNA (NM000926.4). Thin gray lines interspersed in cow transcript represent gaps in the alignment.

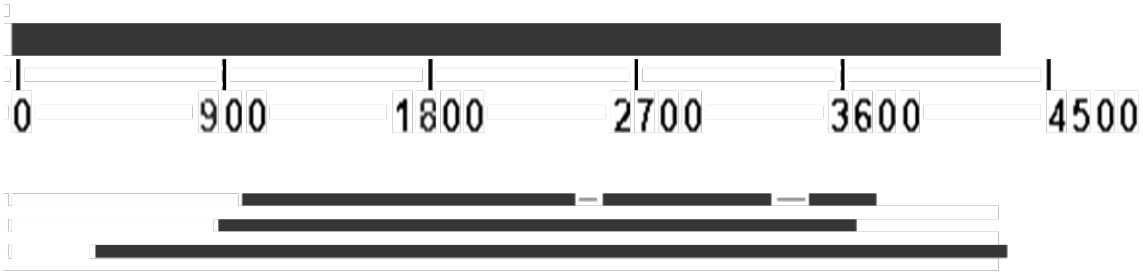

Figure 3.5 Alignment of porcine PGR mRNA (GQ903679.1) and bovine PGR mRNA (XM583951.5) sequences. Alignment generated by BLASTN software (NCBI). Query (top) denotes pig mRNA sequence; Sbjct (bottom) denotes cow mRNA sequence and locations (bases) in the sequence are shown. Nucleic acid residues are listed in FASTA format (A = adenine; T = thymidine; C = cytosine; G = guanine; dashes = gaps; vertical lines = identities). Sequence represented in lowercase, gray letters indicate regions of low sequence complexity. The identity between pig and cow sequence is: A) 1235/1550 (79%); B)723/767 (94%); C) 305/326 (93%). The number of gaps between the sequences is: A) 1/767 (0%); B) 112/1550 (7%); C) 12/326 (3%).

A)

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Query  1029  ATGACTGAGCTGAAGGCAAAGGATCCCCGGGCTCCCCACGTGGCGGGCAGAGCGCCCTCC
             |||||||||||||||| |||| |||||| |||||||||||||||||| |||||||||
Sbjct  1     ATGACTGAGCTGAAGGCGAAGGGTCCCCGCGCTCCCCACGTGGCGGGCACAGCGCCCTCG

Query  1089  CCCACCCA-GCTCGG-G----AC-GCTGGGACGCCCAGACACAGGCCCCTTTCAGGCGAG
              |||||||| || ||| |     | || |||||||||||||| | |||||||| |||||| |
Sbjct  61    CCCACCCAGGC-CGGAGCCCCTCTGCGGGGACGCCCAGACGCGGGCCCCTTCCAGGCGGG

Query  1142  CCAGACCTCGGAAGCGTCGCCCGCAGCCTC-GGCCATACCCCTCTCCCTGGACGGGCT-A
              | || ||||||   || ||||| ||||||| |||| | |||||||||||||||| || |
Sbjct  120   CGAGGCCTCGGGCCCGACGCCCACAGCCTCGGGCC-TCCCCCTCTCCCTGGACGGACTGA

Query  1200  CTCTTCCCTGGGCCCTGCCAGGGACAGGAACC-AGACGGGAAGACGCAGGA-CCAGCAGT
               |||||||| || |||||||||  |||| |||  |||||||||||||| ||| |||| |||
Sbjct  179   -TCTTCCCTCGGTCCTGCCAGGCTCAGG-ACCTGGACGGGAAGACGCCGGACCCAG-AGT

Query  1258  CGCTGTCAGACGTGGAGGGGGCGTATCCCACAGTTGAAGCTACAGAGGGTGCTGGAGGTG
              ||||| |||||||||||||||||||| ||  ||  |||||  ||||||| |||   | |
Sbjct  236   CGCTGGCAGACGTGGAGGGGGCGTATTCCGGAGGGGAAGCCGCAGAGGGCGCT---GCT-

Query  1318  GCAGCTCTAGACCCTCGGAAAAAGACACCGGGCTGCTGGACAGTGTCTTGGACACGCTAC
              || |    |||||| | || || |||  |||| |||||||||| ||| ||||||| || |
Sbjct  292   GC-G----AGACCCCCAGAGAAGGACGGCGGGTTGCTGGACAGCGTCCTGGACACCCTGC

Query  1378  TAGCGCCCTCAGGTCCC-GGGCAGAGCCACGCCAGCCCTC-CCGCCTGCGAAGCCACCAG
              | |||||| | || ||| | || ||||| ||||||||| | ||| |||||||||| ||||
Sbjct  347   TGGCGCCC-CCGGGCCCTGAGCGGAGCCCCGCCAGCCC-CGCCGTCTGCGAAGCCCCCAG

Query  1436  CCCTTGGTGCTTGTTTGGCTCTGAGCTTCCCCAGGACGCTCGGGTTGCCCCTTCCACCCA
              |||||||||| | |||||| | ||||||  ||| ||||| |||| ||| ||  || ||||
Sbjct  405   CCCTTGGTGCCTCTTTGGCCCCGAGCTTGTCCAAGACGCCCGGGCTGCGCCAGCCGCCCA

Query  1496  GGGAGTATTGC-CCCTGCTCATGAGCCGGCCAGAGGGCAAGGCTGGCGACAGCTCCGGGA
              ||| ||  | | |||  |||||||||| |||||| |||||||||||||| | |||||
Sbjct  465   GGGGGTCCTACTCCC-ACTCATGAGCCGCCCAGAGAGCAAGGCTGGCGACACCCCCGGGG

Query  1555  CGGCAGCCGCCCATAAAGTGCTATCCAGGGGTCTGGCACCGTCCCGGCAGCTGCT-GCCC
              | || |||| ||| || |||||  || |||| ||||| || | |||||||||||| ||||
Sbjct  524   CCGCCGCCGGCCAGAAGGTGCTGCCCCGGGGCCTGGCGCCATGCCGGCAGCTGCTGGCCC

Query  1614  TCGACTGCCGGGAGCCATCACTGGCCGGCGGCCGCAG-TGAAGCCCTCTCCGCAGCCCGC
               |||| |||  |||||| | |||||| ||| |||| |  || ||| ||||||||||||||
Sbjct  584   -CGACGGCCTCGAGCCACCCCTGGCCCGCGCCCGC-GCCGAGGCCGTCTCCGCAGCCCGC

Query  1673  CGTGGTGGAGGTGGAGGAGGAGGATGACTCCGAGT-CCGAGGGCTCCAT-GGTTCCACTT
               ||||||||| |   ||||||||| ||||| || | |||||||||| | ||  || |
Sbjct  642   GGTGGTGGAGCT---GGAGGAGGACGACTCGGA-TCCCGAGGGCTCC-TCGGGGCCGCCC

Query  1731  CTGAAGGGTAAACCCCGGCCTGCAGGAGGCACGGCTGCCGGAACAGGAACCCCGACCGGT
              |||||||| ||| ||||||| || ||||| | | | | | || |    |    |  |
Sbjct  697   CTGAAGGGCAAAGCCCGGCCCGCGGGAGG-A-GAC-G-C-GATC----A----G--C---

Query  1791  GCTCCTGGGACGGCCGCAGGAGGCGTCGCCCTGGTCCCCAAGGAAGATGCCCGCTTCTCG
              |||||||||||||||| |||||||||||| ||| ||||| ||||||||||||||||||
Sbjct  739   GCTCCTGGGACGGCCGCGGGAGGCGTCGCCCCGGTGCCCAAAGAAGATGCCCGCTTCTCG
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Query  1851  GCGCCCAGGGGCGCCCTGGCGGAGCACGATGCGTCAGGGGCGCCCGGGCGCTCCCCTCTG
             |||||||||| ||||| ||||||||| || ||  | |  |||||||||||| || |  ||
Sbjct  799   GCGCCCAGGGCCGCCCCGGCGGAGCATGACGCACCCGCCGCGCCCGGGCGCGCCTCGGTG

Query  1911  GCCACTACGGTGATGGATTTTATCCACGTGCCCATCCTGCCGCTCAACACGGCCTTTCTG
             |||||  |||||||||| || |||||||||||||||||||||||||| | ||||| |||
Sbjct  859   GCCACCGCGGTGATGGACTTCATCCACGTGCCCATCCTGCCGCTCAACTCAGCCTTCCTG

Query  1971  GCCGCCCGCACCCGGCAACTGCTAGAGGGGGATAACTATGACGGCGGGGCCCCGGCTGTC
             |||||||||||||||| ||||| ||||| || | ||||||||||||||| |  | |
Sbjct  919   GCCGCCCGCACCCGGCAGCTGCTGGAGGGTGACAGCTATGACGGCGGGGCCGC--C-G--

Query  2031  AGCGCCTTTGCACCGCCGCGGGGCTCGCCCTCGGCCCCGTCCGCC-TCCGTCACCGCC-G
              ||||||| || |||||||||||||||||||| ||| || ||||| |  | | | ||| |
Sbjct  974   -GCGCCTTCGCCCCGCCGCGGGGCTCGCCCTCCGCCTCGGCCGCCCTGGG-CGC-GCCCG

Query  2089  GCGACTTCCCCGACTGCGCGTACTCTGCA-GACGACGAGCCCAAGGACGACGCATTCCCG
             | |||||||||||||||||||| | ||  |||  |||||||||||||||||| ||| ||
Sbjct  1031  GAGACTTCCCCGACTGCGCGTAC-CAGCCCGACTCCGAGCCCAAGGACGACGCGTTCTCG

Query  2148  CTCTATGGCGACTTC-CAGCCGCCCGCCCTGAAGATCAAGGAAGAGGAGGAAGG---CGC
             ||||| || |||  | |||||||||||||||||||||||||| |||||||| ||   |||
Sbjct  1090  CTCTACGGGGACC-CGCAGCCGCCCGCCCTGAAGATCAAGGAGGAGGAGGAGGGGGGCGC

Query  2204  CGAGGCCGCCGCGCGCTCTCCGCGGTCGTACCTGGTGGCGGGTGCCAACCCTGCCGTCTT
             |||||| |||||||||||| |||||| |||||||| ||||||| |||| ||| ||||||||
Sbjct  1149  CGAGGCAGCCGCGCGCTCCCCGCGGCCGTACCTGCTGGCGGGGGCCAGCCCCGCCGTCTT

Query  2264  C-CCGGATTTTCCGCTGGCGCCGCCGTCGCTGCCTCCGCGAGCGTCGTCCAGACCTGG-G
             | ||| | || |||||||||||||||| | | | | | | | |  |   ||   ||| |
Sbjct  1209  CGCCG-ACTTCCCGCTGGCGCCGCCGTC-CAGGC-C-G-G-G-G--G---AGG--TGGCG

Query  2322  GAAGCCTCGGCGGCGGTCGCACCCACCAGTGCCTC-GGTATCGTCGGCGTCC-TCGTCGG
             |  ||| || ||||   ||| |  ||  | ||| | ||  ||||| ||| || | |||||
Sbjct  1255  GT-GCC-CG-CGGC---CGC-C--ACGGGGGCCCCCGGC-TCGTCCGCGGCCGT-GTCGG

Query  2380  GGTCGGCCTTGGAGTGCATGTTGTATAAGGCGGAGGGCGCGCCGCCCCAACAGGGCCCGT
             |||| ||| |||||||| |  |||| |||||||||||||||||||| | ||||||||
Sbjct  1304  GGTCAGCCCTGGAGTGCGTCCTGTACAAGGCGGAGGGCGCGCCGCCCCAGCCGGGCCCGT

Query  2440  TCCCGCCTCCGCCCTGCAAGCCGCCGAGc-gccggcgcctgcctgctgccgcgggacagc
             || |||| |||||||||||| | ||| || || ||||||||||||||||||||||||  |
Sbjct  1364  TCGCGCCGCCGCCCTGCAAGGCTCCG-GCCGCAGGCGCCTGCCTGCTGCCGCGGGACNNC

Query  2499  ctgcc-gtccacctcggcctccgccgccgcc-gccgccgccgcc-gccgc  2545
              |||| |||||||||||| |||||||||| |||||||  ||| |||||
Sbjct  1423  -TGCCCGTCCACCTCGGCCGCCGCCGCCGCCCGCCGCCGGGGCCTGCCGC  1471
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B)

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Query  2627  GGGCCTGCCGCAGGTGTACCAGCCCTATCTCAACTACCTGAGGCCGGATTCAGAAGCCAG
             ||||||||||||||| |||||||||||||||||||||| ||||||||||||||||||||
Sbjct  1461  GGGCCTGCCGCAGGTCTACCAGCCCTATCTCAACTACCTTAGGCCGGATTCAGAAGCCAG

Query  2687  CCAGAGCCCACAGTACAGCTTCGAGTCATTACCTCAGAAGATTTGTTTAATCTGTGGGGA
             |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  1521  CCAGAGCCCACAGTACAGCTTTGAGTCATTACCTCAGAAGATTTGTTTAATCTGTGGGGA
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Query  2747  TGAAGCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAA
             |||||||||||||||||||||||||||||| ||||||||| ||||||||||||||||||||
Sbjct  1581  TGAAGCATCAGGCTGTCATTATGGTGTCCTCACCTGTGGAAGCTGTAAGGTCTTCTTTAA

Query  2807  AAGGGCAATGGAAGGGCAGCACAACTACTTATGTGCTGGAAGAAACGACTGCATTGTTGA
             ||||||||||||||| ||||| ||||||||||||||||||||||| ||||||||||||
Sbjct  1641  AAGGGCAATGGAAGGACAGCATAACTACTTATGTGCTGGAAGAAACGATTGCATTGTTGA

Query  2867  TAAAATCCGCAGAAAAAACTGCCCAGCTTGTCGCCTTAGAAAGTGCTGTCAGGCTGGCAT
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  1701  TAAAATCCGCAGAAAAAACTGCCCAGCATGTCGCCTTAGAAAGTGCTGTCAGGCTGGCAT

Query  2927  GGTCCTTGGAGGTCGAAAGTTTAAAAAGTTCAATAAAGTTAGAGTTATGAGAGCACTAGA
             ||| |||||||| ||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  1761  GGTTCTTGGAGGCCGAAAGTTTAAAAAGTTCAATAAAGTTAGAGTTATGAGAACACTAGA

Query  2987  TGCCGTTGCTCTCCCACAGTCGGGGGGCATTCCAAATGAAAGTCAGGCCCTAAGCCAGAG
             ||| ||||| ||||||||| ||| ||| |||||||| ||||| || ||||||||||||||
Sbjct  1821  TGCTGTTGCCCTCCCACAGCCGGTGGGTATTCCAAACGAAAGCCAAGCCCTAAGCCAGAG

Query  3047  AATCACTTTTTCACCAAATCAAGACTTACAGCTGATCCCCCCGTTGATCAACTTGCTCAT
             ||||||||||||||| ||||||||| ||| ||||||||||  |||||||||||||||||
Sbjct  1881  AATCACTTTTTCACCAAGTCAAGACTTGCAGTTGATCCCCCCACTGATCAACTTGCTCAT

Query  3107  GAGCATTGAACCAGATGTGGTCTATGCAGGACATGACAACACCAAACCCGACACTTCGAG
             |||||||||||||||||||||||||||||||||||||||| |||||| || || || ||
Sbjct  1941  GAGCATTGAACCAGATGTGGTCTATGCAGGACATGACAACAGCAAACCTGATACCTCCAG

Query  3167  TTCTTTGCTGACAAGTCTGAATCAGCTGGGTGAAAGACAACTTCTTTCAGTAGTCAAGTG
              ||| |||||||||||||||||||| ||||| |||||||||||||||| ||||||||
Sbjct  2001  CTCTCTGCTGACAAGTCTGAATCAGCTTGGTGAGAGACAACTTCTTTCAGTGGTCAAGTG

Query  3227  GTCTAAGTCACTGCCAGGTTTTCGAAACTTACATATTGATGACCAAATAACTCTCATCCA
             |||||| |||||||||||||||| |||||||||||||||||||||||||||||||||||
Sbjct  2061  GTCTAAATCACTGCCAGGTTTTCGGAACTTACATATTGATGACCAAATAACTCTCATCCA

Query  3287  GTATTCCTGGATGAGCTTAATGGTATTTGGACTAGGATGGAGATCCTATAAACATGTCAG
             |||||| |||||||| |||||||||||||||||||||||| ||||| ||||||||
Sbjct  2121  GTATTCTTGGATGAGTTTAATGGTATTTGGACTAGGATGGAGATCTTATAAGCATGTCAG

Query  3347  TGGACAGATGCTTTATTTTGCACCTGATCTAATACTAAATGAACAGC  3393
             |||||||||||| |||||||| |||||||||||||||||||| |||
Sbjct  2181  TGGACAGATGCTATATTTTGCGCCTGATCTAATACTAAATGAA-AGC  2226
```

C)

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Query  3520  TTCCTTTGGAAGGACTAAGAAGTCAAAACCAGTTTGAAGAGATGAGATCAAGCTACATTA
             ||||||| |||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  2363  TTCCTTTAGAAGGACTAAGAAGTCAAAACCAGTTTGAAGAGATGAGATCAAGTTACATTA

Query  3580  GAGAGCTCATCAAGGCAATTGGTTTGAGACAAAAAGGAGTCGTCCCTAGCTCACAGCGTT
             |||||||||||||||||||||||||| |||||||||| ||||||||||||||||||||
Sbjct  2423  GAGAGCTCATCAAGGCAATTGGTTTGAGGCAAAAAGGAGTTGTCCCTAGCTCACAGCGTT
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Query  3640  TCTACCAGCTTACAAAACTTCTTGATAACTTGC-----AT--G-A---T-CTTGTCAAAC
             |||| ||||||||||||||||||||||||||     ||  | |   | ||||||||||
Sbjct  2483  TCTATCAGCTTACAAAACTTCTTGATAACTTGCATGATATAAGTATTTTGCTTGTCAAAC

Query  3688  AACTTCATCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAATTTC
             |||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  2543  AACTTCATCTGTATTGCTTGAATACATTTATCCAGTCCTGGGCACTGAGTGTTGAATTTC

Query  3748  CAGAAATGATGTCTGAGGTTATTGCTGCACAATTACCCAAGATCTTGGCAGGGATGGTGA
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2603  CAGAAATGATGTCTGAGGTTATTGCTGCACAATTACCCAAGATCTTGGCAGGGATGGTGA

Query  3808  AACCCCTTCTCTTTCATAAAAAGTGA  3833
             || | ||||||||||||||||||||
Sbjct  2663  AATCTCTTCTCTTTCATAAAAAGTGA  2688
```

Figure 3.6 Alignment of porcine PGR mRNA (GQ903679.1) and murine PGR mRNA (NM008829.2) sequences. Alignment generated by BLASTN software (NCBI). Query (top) denotes pig mRNA sequence; Sbjct (bottom) denotes mouse mRNA sequence and locations (bases) in the sequence are shown. Nucleic acid residues are listed in FASTA format (A = adenine; T = thymidine; C = cytosine; G = guanine; dashes = gaps; vertical lines = identities). Sequence represented in lowercase, gray letters indicate regions of low sequence complexity. The identity between pig and mouse sequences is 2377/2989 residues (79%) with 5% gaps between the sequences (176/2989).

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Query  921   ACACCTATTTTctcctcccgctgcccctatattc-ccgacacccccctcatccttccccta
             ||||| | || |||||||| |||||||||||  || ||| |||||||||  ||||||||  |
Sbjct  528   ACACCCACTTCCTCCTCCCTCTGCCCCTATA-TCACCGGCACCCCCTCCTCCTTCCCTTT

Query  980   tccctcctccccA-GAGACAGGGGAGGAGAAAAGGGGAGTC-CAGGTCGTCATGACTGAG
             |||||||| |||| |||||||||||||||||||||||| |   ||||||||||||||||
Sbjct  587   TCCCTCCT-CCCAGGAGACAGGGGAGGAGAAAAGGGGAG-CTTGGGTCGTCATGACTGAG

Query  1038  CTGAAGGCAAAGGATCC-CCGG-GCTCCCCACGTGGCGGGCAG-AGCGCCCTCCCCCACC
             ||| |||||||||||| | ||  |||| |||||  | ||| |   |||||||||| ||
Sbjct  645   CTGCAGGCAAAGGATCCGCAGGTTCTCCACACGT--CTGGC-GCTTCGCCCTCCCCC-CC

Query  1095  -CAGC-TCGGG----AC--GCTGGGACGCCCAGACACAGGCCCCTTTC-AGGCGAGCCAG
              || | |||||     |  ||| | ||||   ||| |||| ||||| | ||| |||||||
Sbjct  701   ACA-CATCGGGTCCCCCTTGCTTGCACGCTTGGACTCAGGTCCCTTCCAAGG-GAGCCAG

Query  1146  AC-CTCGGAAGCGTCGCCCGCAGCCTCGGCC-ATACCCCTCTCCCTGGACGGGCTACTCT
              | |||||| | |||| | | || ||| ||| |||||  |||||||||||||||| || |
Sbjct  759   -CACTCGGACGTGTCGTCTGTAGTCTC-GCCTATACCGATCTCCCTGGACGGGCTGCTTT

Query  1204  TCCCTGGGCCCTGCCAGGG--AC-AGGAACCAGACGGGAAGAC-GCAGGACCAGCAGTCG
             | ||| || |||||| |||   | ||   |||||||| ||||| |  ||||||||||||
Sbjct  817   TTCCTCGGTCCTGCCGGGGTCCCGAGCTCCCAGACGGAAAGACAG-GGGACCAGCAGTCG

Query  1260  CTGTCAGACGTGGAGGGGGCGTATC-CCACAGTTGAAGCTA--CAGAGGGTGCTGGAGGT
             ||||| |||||||||| || | || |    || ||||| |  || | || |  |||||
Sbjct  876   CTGTCCGACGTGGAGGGAGC-TTTCTCTGGGGTAGAAGCCACTCATA-GG-GAAGGAGGC

Query  1317  GGCAGCTCTAGA-CCCTCGGAAAAAGACACCGGGCTGC-TGGACAGTGTCTTGGACACGC
              | |  || ||| ||| |||| || |||| | | || | | |||||||||| ||| ||
Sbjct  933   AGAAATTCCAGAGCCC-CGGAGAAGGACAGCAGACT-CTTAGACAGTGTCTTAGACTCG-

Query  1375  TACTAG-CGCCCTCAGGTCCCGGGCAGAGCCACGCCAGCCCTCCCGCCTGCGAAGCCACC
             |  | | | ||||| ||  |||  ||||| |||||||||||||| |||||||| |||| |
Sbjct  990   TTGTTGACTCCCTCCGGGACCGAACAGAGTCACGCCAGCCCTCCAGCCTGCGAGGCCATC

Query  1434  AGC--CCTTGGTGCT-TGTTT-GGCTCTGAGCTTCCCCAGGACGCTCG-GGTTGCCCCTT
             | |  || ||||| | | ||| ||| | ||||||||  | ||| | ||  | || ||||
Sbjct  1050  A-CTTCC-TGGTG-TCTCTTTGGGC-CAGAGCTTCCAGAAGACCCCCGCAG-TGTCCCTG

Query  1489  CCACC-CAGGGAGTATTG-CCCCTGCTCATGAGCCGGCCAGAGGGCAAGGCTGGCGACAG
             | |||  ||||  | ||| |||| ||||||||| |||||||||  ||||||||||||||
Sbjct  1105  CTACCAAAGGG-TTGTTGTCCCC-GCTCATGAGTCGGCCAGAGATCAAGGCTGGCGACAG

Query  1547  CTCCGGGAC-GGCAGCCGCCCATAAAGTGCTATCCAGGGGTCTGGCACCGTCCC-GGCAG
             ||||||||| || ||| |  || || |||||  |||  || ||| ||||  ||| |||||
Sbjct  1163  CTCCGGGACAGG-AGCAGGACAGAAGGTGCTGCCCAAAGGACTGTCACC-ACCCAGGCAG

Query  1605  CTGCTGC-CCTCGA-CTGCCGGGAG-CCATCACTGG-CCGGCGGCCGCAGTGAAGCCCTC
             ||| ||| || | | || | |||||  | ||||||| |||| ||| |  |||||||| ||
Sbjct  1221  CTGTTGCTCC-CTACCT-CGGGGAGTGC-TCACTGGCCCGG-GGCAGGGGTGAAGCCGTC

Query  1661  TCCGCAGCCCGCCG-TGGTGGAGGTGGAGGAGGAGGA-T-GACTCCGAGTCCGAGGGCTC
              |||||||| || |  || ||||||||| |||||  | | | ||  ||| |||||||||
Sbjct  1277  CCCGCAGCCAGCTGCAGG-GGAGGTGGAAGAGGA-CAGTGGCCT-GGAGACCGAGGGCT-

Query  1718  CATG--GTTCCACTTCTGAAGGGTAAACCCCG-GC-CTGCAGGAGGCA-CGGCTGCCGGA
             | ||  | ||| ||||| ||| | ||||| || || |||  | ||||| |||| | ||||
Sbjct  1333  C-TGCAGCTCCGCTTCTAAAGAGCAAACCTCGAGCACTG--GAAGGCACCGGCAG-CGGA
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Query  1773  ACAGGA-ACCCCGACCGGTGC-TCCTGGGACGGCCGCAGGAGGCGTCGCCCTGGTCCCCA
               ||||  | | | ||   ||  |||  | ||||| |||| || ||| | ||||||||  |
Sbjct  1389  GGAGGAGTCGCAG-CCAACGCGGCCTCAG-CGGCCCCAGGCGGTGTCACTCTGGTCCCAA

Query  1831  AGGAAGATGCCCGCTTCTCGGCGCCCAGGGGCGCCCTGGCGGAGCACGA-TGCGT--CAG
              |||||||| | || || || || || |||| |  |||   |||||| || | | |  ||
Sbjct  1447  AGGAAGATTCACGGTTTTCTGCTCCTAGGGTC-TCCT--TGGAGCAAGACT-C-TCCCA-

Query  1888  GGGCGCCCGGGCGCTCCCCTCTGGCCACTACGGTGATGGATTTTATCCACGTGCCCATCC
               || |||||||||||||| |||||||| || ||| ||||||| ||||| ||||||||||
Sbjct  1501  TTGCCCCCGGGCGCTCCCCACTGGCCACCACAGTGGTGGATTTCATCCATGTGCCCATCC

Query  1948  TGCCGCTCAA-CACGGC-CTTTCTGGCCGCCCGCACCCGGCAACTGCTAGAGGGGGATAA
              |||| || || ||| || | | ||||||||||||||||||||| ||||| |||||||| |
Sbjct  1561  TGCCTCTGAACCAC-GCAC-TCCTGGCCGCCCGCACCCGGCAGCTGCTGGAGGGGGACAG

Query  2006  CTATGACGGCGGGGCCCCGGCTGTCAGCG-CC-TTTGCACCGCCGCGGGGCTCGCCCTCG
              ||| |||||||||||| | ||   ||| | || ||||| |||||  ||||||||||||
Sbjct  1619  CTACGACGGCGGGGCCACAGC--CCAGGGCCCTTTTGCCCCGCCTAGGGGCTCGCCCTCC

Query  2064  GCCCCGTCCGCCTCC-GT-CACC-GCCGGCGACTTCCCCGACTGCGCGTA--CTCTGCAG
              || || ||| || || || | || | ||| |||||||| |||||| | ||  |||||  |
Sbjct  1677  GCGCCATCC-CCGCCGGTGC-CCTG-CGGTGACTTCCCAGACTGCACCTACCCTCTG--G

Query  2119  ACGACGAGCCCAAGGACGACGCATTCCCGCTCTATGGCGACTTCCAGCCGCCCGCCCTGA
              | | ||| ||||| || ||||  ||||| || || |||||||||||| |||| | | |||
Sbjct  1732  AAGGCGACCCCAAAGAGGACGTGTTCCCTCTTTACGGCGACTTCCAGACGCCTGGCTTGA

Query  2179  AGATCAAGGAAGAGGAGGAAGGCGCCGAGGCCGCCGCGCGCTCTCCGCGGTCGTACCTGG
              |||||||||| |||||||| ||||| || || || | |||||| |||||  | ||||| |
Sbjct  1792  AGATCAAGGAGGAGGAGGAGGGCGCGGATGCTGCTGTGCGCTCGCCGCGCCCCTACCT-G

Query  2239  T-GGCGGGTGCCAAC-CCTGCCGTCTTCCCGGATTTTCCGCTGGCGCCGCCGTCGCTGCC
              | ||| || |||| | ||| ||  |||||| || || ||||| | | | ||  ||| |||
Sbjct  1851  TCGGCTGGAGCCAGCTCCT-CCACCTTCCCAGACTTCCCGCT-G-G-CACC--CGC-GCC

Query  2297  TCCGCGAGCG---TCGTCCAGACCTGGGGAAGCCTCGGCGG-CGGTCGCACCCACCAGTG
               | |||||||   || ||||| || || |||||  ||| || ||| || ||||| ||| |
Sbjct  1904  GCAGCGAGCGCCATCCTCCAGGCCCGGAGAAGCGGCGGTGGCCGG-CGGACCCAGCAGCG

Query  2353  CCTCGGTATCGTCGGCGTCCTCGTCGGGGTCGGC-CTTGGAGTGCATGTTGTATAAGGCG
              || |||| ||| | |||||||| || || || || | ||||||||||  |||| || |||
Sbjct  1963  CCGCGGTGTCGCCAGCGTCCTCCTCCGGCTCCGCGC-TGGAGTGCATCCTGTACAAAGCG

Query  2412  GAGGGCGCGCCGCCCCA-ACAGGGCCCGTTCCCGCCTCCGCCCTGCAAGCCGCCGAG-cg
              |||||||||||| ||||  |||||  ||||| |||| | ||| |||||||| || || ||
Sbjct  2022  GAGGGCGCGCCG-CCCACGCAGGGTTCGTTCGCGCCACTGCCGTGCAAGCC-CCCAGCCG

Query  2470  ccggcgcctgcctgctgccgcgggacagcctgccgtccacctcggcctccgccgccgccg
              ||||| ||||||| || || ||||||||||   |     | |||   ||||| |||  |
Sbjct  2080  CCGGCTCCTGCCTACTACCCCGGGACAGCCT---G-----C-CGG---CCGCC-CCG--G

Query  2530  ccgccgccgccgccgccgGGGCGGCTCCTGCGCTCTACCAGCCGCTTGGCCTTAACGGGC
              || ||||||| | | ||    | |   |  |  |||||||||||| ||||| || ||||
Sbjct  2125  CCACCGCCGCAG-CACC----C-G---C--C-ATCTACCAGCCGCTCGGCCTCAATGGGC

Query  2590  TCCCGCAGCTCGGCTACCAGGCCGCGGTACTCAAGGAGGGCCTGCCGCAGGTGTACCAGC
              |||||||||| |||||||||||||||| ||||||||  ||||||| ||||| |||| ||
Sbjct  2173  TCCCGCAGCTGGGCTACCAGGCCGCGGTGCTCAAGGACAGCCTGCCCCAGGTCTACCCGC
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Query  2650  CCTATCTCAACTACCTGAGGCCGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTCG
             | || || ||||||||||||| ||||||||||||||||||||||||||||  |||| |
Sbjct  2233  CATACCTTAACTACCTGAGGCCAGATTCAGAAGCCAGCCAGAGCCCACAGTATGGCTTTG

Query  2710  AGTCATTACCTCAGAAGATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATG
             | || ||||||||||||| || |||||||| ||||||||||||| |||||||| ||||
Sbjct  2293  ATTCCTTACCTCAGAAGATCTGCTTAATCTGCGGGGATGAAGCATCTGGCTGTCACTATG

Query  2770  GTGTCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAAAAGGGCAATGGAAGGGCAGCACA
             | || |||||||||||||||| ||||||||||||| |||||||||||||||||||| |
Sbjct  2353  GCGTGCTTACCTGTGGGAGCTGCAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCATA

Query  2830  ACTACTTATGTGCTGGAAGAAACGACTGCATTGTTGATAAAATCCGCAGAAAAAACTGCC
             |||| |||||||||||||||| |||||||||||||||||||| ||||||||||||||||
Sbjct  2413  ACTATTTATGTGCTGGAAGAAATGACTGCATTGTTGATAAAATTCGCAGAAAAAACTGCC

Query  2890  CAGCTTGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTCCTTGGAGGTCGAAAGTTTA
             |||| ||||| || |||||||| |||||||||||||||||||||||||||| |||||||
Sbjct  2473  CAGCATGTCGTCTGAGAAAGTGTTGTCAGGCTGGCATGGTCCTTGGAGGTCGTAAGTTTA

Query  2950  AAAAGTTCAATAAAGTTAGAGTTATGAGAGCACTAGATGCCGTTGCTCTCCCACAGTCGG
             | ||||| ||||||||  |||||||||||  | || || |  |||||||||||  |||||||
Sbjct  2533  AGAAGTTTAATAAAGTCCGAGTTATGAGAACCCTTGACGGTGTTGCTCTCCCCCAGTCGG

Query  3010  GGGGCATTCCAAATGAAAGTCAGGCCCTAAGCCAGAGAATCACTTTTTCACCAAATCAAG
              |||| |||| || || || ||||||||  |||||||||||||| ||||||||||||||||
Sbjct  2593  TGGGCCTTCCTAACGAGAGCCAGGCCCTGGGCCAGAGAATCACCTTTTCACCAAATCAAG

Query  3070  AC-TTACAGCTGATCCCCCCGTTGATCAACTTGCTCATGAGCATTGAACCAGATGTGGTC
             |  || || ||| |||| ||  | |||||| |||||||||||||||| || |||||||||
Sbjct  2653  AAATT-CAACTGGTCCCGCCACTCATCAACCTGCTCATGAGCATTGAGCCTGATGTGGTC

Query  3129  TATGCAGGACATGACAACACCAAACCC-GACACTTCGAGTTCTTTGCTGACAAGTCTGAA
             |||||||| |||||||||| ||| || |||||||| || |||||||||| ||||| ||
Sbjct  2712  TATGCAGGGCATGACAACAC-AAAGCCTGACACTTCCAGCTCTTTGCTGACCAGTCTCAA

Query  3188  TCAGCTGGGTGAAAGACAACTTCTTTCAGTAGTCAAGTGGTCTAAGTCACTGCCAGGTTT
              || || || || |||||||| ||||||||||||| |||||||| || ||||||||||
Sbjct  2771  CCAACTAGGCGAGAGACAACTGCTTTCAGTAGTCAAATGGTCTAAATCTCTGCCAGGTTT

Query  3248  TCGAAACTTACATATTGATGACCAAATAACTCTCATCCAGTATTCCTGGATGAGCTTAAT
              || |||||||| |||||||||| ||||| || || ||||| |||||||||||| | ||
Sbjct  2831  CCGGAACTTACACATTGATGACCAGATAACCCTGATTCAGTACTCCTGGATGAGCCTGAT

Query  3308  GGTATTTGGACTAGGATGGAGATCCTATAAACATGTCAGTGGACAGATGCTTTATTTTGC
             ||| ||||| || || ||||| || || || |||||||||||||||||||| ||||||||
Sbjct  2891  GGTGTTTGGCCTGGGGTGGAGGTCGTACAAGCATGTCAGTGGACAGATGCTATATTTTGC

Query  3368  ACCTGATCTAATACTAAATGAACAGCGGATGAAGGAA-TCATCATTCTATTCATTATGCC
             |||||||||||| |||||||| ||| ||||||||||  |  |||||||| ||  | ||||
Sbjct  2951  ACCTGATCTAATCCTAAATGAGCAGAGGATGAAGGAGCTG-TCATTCTACTCGCTGTGCC

Query  3427  TTACCATGTGGCAGATCCCACAGGAGTTTGTGAAGCTTCAAGTTAGCCAAGAAGAGTTTC
             |||||||||||| |||||||||||||||| || || || || | ||| || || || |
Sbjct  3010  TTACCATGTGGCAAATCCCACAGGAGTTTGTCAAACTCCAGGTGACCCATGAGGAATTCC

Query  3487  TCTGTATGAAAGTATTGCTACTTCTTAATACAATTCCTTTGGAAGGACTAAGAAGTCAAA
             |||||||||||| || |||||||||| |||||||||||||||||||| || |||||||
Sbjct  3070  TCTGTATGAAAGTCTTACTACTTCTTAACACAATTCCTTTGGAAGGACTGAGGAGTCAAA
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Query  3547  ACCAGTTTGAAGAGATGAGATCAAGCTACATTAGAGAGCTCATCAAGGCAATTGGTTTGA
              ||||||||||||||||||||||||||| ||  | ||  | |||||||||||||||| |
Sbjct  3130  GCCAGTTTGAAGAGATGAGATCAAGCTATATCCGCGAATTGATCAAGGCAATTGGTTTAA

Query  3607  GACAAAAAGGAGTCGTCCCTAGCTCACAGCGTTTCTACCAGCTTACAAAACTTCTTGATA
              |||| ||||| || ||||| |||||||||||| |||||||| || |||||||||||| || |
Sbjct  3190  GACAGAAAGGGGTTGTCCCCAGCTCACAGCGCTTCTACCAACTCACAAAACTTCTCGACA

Query  3667  ACTTGCATGATCTTGTCAAACAACTTCATCTGTACTGCTTGAATACATTTATCCAGTCCC
               ||||||||||||||| ||||| || || |||||||||||||||||||| ||||| ||||
Sbjct  3250  GCTTGCATGATCTTGTGAAACAGCTCCACCTGTACTGCTTGAATACATTCATCCAATCCC

Query  3727  GGGCACTGAG-TGTTGAATTTCCAGAAATGATGTCTGAGGTTATTGCTGCACAATTACCC
              || ||||| | ||| |||||||| |||||||||||||| |||||||||| || || |||
Sbjct  3310  GGACACTG-GCTGTGGAATTTCCGGAAATGATGTCTGAAGTTATTGCTGCCCAGTTGCCC

Query  3786  AAGATCTTGGCAGGG-ATGGTGAAACCCCTTCTCTTTCATAAAAAGTGA  3833
              |||||| |||| ||| |||||||| || || |||||||| |||||||||
Sbjct  3369  AAGATCCTGGC-GGGCATGGTGAAGCCGCTCCTCTTTCACAAAAAGTGA  3416
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Figure 3.7 Alignment of porcine PGR mRNA (GQ903679.1) and human PGR mRNA (NM000926.1) sequences. Alignment generated by BLASTN program (NCBI). Query (top) denotes pig mRNA sequence; Sbjct (bottom) denotes human mRNA sequence and locations (bases) in the sequence are shown. Nucleic acid residues are listed in FASTA format (A = adenine; T = thymidine; C = cytosine; G = guanine; dashes = gaps; vertical lines = identities). Sequence represented in lowercase, gray letters indicate regions of low sequence complexity. The identity between pig and mouse sequences is 3588/4256 residues (84%) with 5% gaps between the sequences (221/4256).

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Query  352   GCATTTTCAGTGAGAACTAGTC-TCACTTGGCATTTGAGTGGAATCTGC-ACCTAC-AGG
             ||||| ||| |||||||||| | ||||||| |||||||||| ||||| | |||  | |||
Sbjct  56    GCATTCTCAATGAGAACTAG-CTTCACTTGTCATTTGAGTGAAATCTACAACC--CGAGG

Query  409   CGTTTCGTCCCCTCCGGACTGCTAAGAAATGTGAGGGCTGCGGAGATGACTGTCGCCAGC
             ||  | || | | ||| ||| ||  |  || ||||  || |||||||||||||||| ||
Sbjct  113   CGGCTAGTGCTC-CCGCACTACT--GGGATCTGAGATCTTCGGAGATGACTGTCGCCCGC

Query  469   TA-TAGGGAACCGGGAGGTA-TAAGACTGTT-CAGGGAATGGGCTGTGCCGAGAGG-CCA
              | || ||| || | | | | |  |||  || | |||||||||||| |||||||| ||
Sbjct  170   -AGTACGGAGCCAGCA-GAAGTCCGACCCTTCCTGGGAATGGGCTGTACCGAGAGGTCC-

Query  525   AAATAGCCCCAGGGTTTTAGGGAGACGGGAAATGTAACACAGGC-AGGGACT--G-GCAG
              | ||||||||||||||||| |||  ||| | || ||| || || |||||||  | ||
Sbjct  227   GACTAGCCCCAGGGTTTTAGTGAG-GGGGCAGTGGAACTCA-GCGAGGGACTGAGAGCTT

Query  581   CA-A-CA-GGGC-A--TTGAAGCCGGAGAAAAAGTCGGGAGAT--A-GAGCCGC----CA
             || | || |  | |  |||| ||| |||||||||||||||||  | |||||||    ||
Sbjct  285   CACAGCATGCACGAGTTTGATGCCAGAGAAAAAGTCGGGAGATAAAGGAGCCGCGTGTCA

Query  628   GT-AATTGCTTTCGAAGCCGCCGCCACTCGAGTG-CTGACTTGTGAGTACTCTGCGTCTC
              | ||||||  ||| |||||| ||||||| |||| | |||||||||||||||||||||||
Sbjct  345   CTAAATTGCCGTCGCAGCCGCAGCCACTCAAGTGCCGGACTTGTGAGTACTCTGCGTCTC

Query  686   CTGTCTTCAGACTGAAGTCGGGGAACTCTCTTGGAGAACTCTCCC-AGTTAGGAAACGAG
             | ||| || ||| ||||| || |||||||||||||||||||| ||| |||||||| |||||
Sbjct  405   CAGTCCTCGGACAGAAGTTGGAGAACTCTCTTGGAGAACTC-CCCGAGTTAGGAGACGAG

Query  745   ATC-CCT-AC-A--ACTACCTTCGCCGGCTCTCCCCACCTG-C-CT--CAAGGACGTAAC
             ||| ||| || |  ||||| ||  |  || |||||||| || | ||  |  ||||  |||
Sbjct  464   ATCTCCTAACAATTACTACTTTTTCTTGCGCTCCCCACTTGCCGCTCGCTGGGAC-AAAC

Query  796   AACTA-TCACCCATCTCCTGCCTACCA-GACGCTGGGCAAGGGCAGGAGCTGA-CAG-G-
              || |  |||   || |||| | | || || |  || |||||||||||||||| ||| |
Sbjct  523   GAC-AGCCACAGTTCCCCTGACGA-CAGGATGGAGGCCAAGGGCAGGAGCTGACCAGCGC

Query  851   TG-CC-GCCCC-CTTCCGACCCAGGAGGTGGAGATTTCACCCCCCTCTCCACCGTCCCGT
              | ||  |||| |  |||||||||||||||||  |      || ||   |||    ||
Sbjct  581   CGCCCTCCCCCGCCCCCGACCCAGGAGGTGGAGA--T------CC-CT---CCG----GT

Query  908   CCAGCCAAATTCAACACCTATTTTctcctcccgctgcccctatattcccgacacccccctc
             ||||||| |||||||||| | |||||||||| |||||||||||||||||| ||||||||
Sbjct  625   CCAGCCACATTCAACACCCACTTTCTCCTCCCTCTGCCCCTATATTCCCGAAACCCCCTC

Query  968   atccttcccctatccctcctccccAGAGACAGGGGAGGAGAAAAGGGGAGTCCAGGTCGT
              |||||||| | |||||||| ||  ||||| ||||||||||||||||||||| |||||
Sbjct  685   CTCCTTCCCTTTTCCCTCCT-CCTGGAGACGGGGGAGGAGAAAAGGGGAGTCCA-GTCGT

Query  1028  CATGACTGAGCTGAAGGCAAAGGATCCCCGGGCTCCCCACGTGGCGGGCAGAGCGCCCTC
             ||||||||||||||||||||| ||||||||||||||||||||||||| |  |||||||
Sbjct  743   CATGACTGAGCTGAAGGCAAAGGGTCCCCGGGCTCCCCACGTGGCGGGCGGCCCGCCCTC

Query  1088  CCCC-A-CCCAGCTCGGGAC-GCTGGGACGCCCAGACACAGGCCCCTTTCAGGCGAGCCA
             |||| |   | | ||   || |||| | ||||||| | |||| || || | || ||||||
Sbjct  803   CCCCGAGGTCGGATCCCCACTGCTGTGTCGCCCAGCCGCAGGTCCGTTCCCGGGGAGCCA

Query  1145  GACCTCGGA-AGCGTCGCCCGCAGCCTCGGCCATACCCCTCTCCCTGGACGGGCTACTCT
             ||||||||| | | | ||| | ||  ||||||||||  ||||||||||||||||||||
Sbjct  863   GACCTCGGACA-CCTTGCCTGAAGTTTCGGCCATACCTATCTCCCTGGACGGGCTACTCT
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Query  1204  TCCCTGGGCCCTGCCAGGGACAGGA--AC-CAGACGGGAAGACGCAGGACCAGCAGTCGC
             ||||| ||||||||||||||||||    | | ||||  ||||||||||||||||||||||
Sbjct  922   TCCCTCGGCCCTGCCAGGGACAGGACCCCTCCGACGAAAAGACGCAGGACCAGCAGTCGC

Query  1261  TGTCAGACGTGGAGGGGGCGTATCCCACAGTTGAAGCTAC-AGAGGGTGCTGGAGGTGGC
             |||| |||||||||||  || ||| ||| || |||||||| || ||||||||||||  ||
Sbjct  982   TGTCGGACGTGGAGGGCGCATATTCCAGAGCTGAAGCTACAAG-GGGTGCTGGAGGCAGC

Query  1320  AGCTCTAGACCCTCGGAAAAAGACACCGGGCTGCTGGACAGTGTCTTGGACACGCTACTA
             || ||||| ||| | ||||| |||| ||| ||||||||||||||||||||||| ||  |
Sbjct  1041  AGTTCTAGTCCCCCAGAAAAGGACAGCGGACTGCTGGACAGTGTCTTGGACACTCTGTTG

Query  1380  GCGCCCTCAGGTCCCGGGCAGAGCC-ACGCCAGCCCTCCCGCCTGCGAAGCCACCAGCCC
             |||||||||||||||||||||||| || ||||||||||||||||||| | ||||||| |
Sbjct  1101  GCGCCCTCAGGTCCCGGGCAGAGCCAAC-CCAGCCCTCCCGCCTGCGAGGTCACCAGCTC

Query  1439  TTGGTGCTTGTTTGGCTCTGAGCTTCCCCAGGA-CGCTCGGGTTGCCCCTTCCACCCAG-
             ||||||| |||||||| | || |||||| | || | | | || ||||||  ||||||||
Sbjct  1160  TTGGTGCCTGTTTGGCCCCGAACTTCCCGAAGATC-CACCGGCTGCCCCCGCCACCCAGC

Query  1497  GGAGTATTG-CCCCTGCTCATGAGCCGG-CCAGAGG-GCAAGGCTGGCGACAGCTCCGGG
             || || ||| |||| |||||||||||| || | || |||||| ||| ||||||||||||
Sbjct  1219  GG-GTGTTGTCCCC-GCTCATGAGCCGGTCC-G-GGTGCAAGGTTGGAGACAGCTCCGGG

Query  1554  ACGGCAGCCGCCCATAAAGTGCTATCCAGGGGTCTGGCACC-GTCCCGGCAGCTGCTGC-
             |||||||| |||||||||||||  || |||| ||| |||| | |||||||||||||||
Sbjct  1275  ACGGCAGCTGCCCATAAAGTGCTGCCCCGGGGCCTGTCACCAG-CCCGGCAGCTGCTGCT

Query  1612  CCTC-GACTGCCGGGAGCCATCACTGG-CCGGCGGCCGCAGTGAAGCCCTCTCCGCAGCC
             || | | || | | ||||| ||||||| |||| |||| |||||||||| ||||||||| |
Sbjct  1334  CC-CGGCCT-CTGAGAGCCCTCACTGGTCCGG-GGCCCCAGTGAAGCCGTCTCCGCAGGC

Query  1670  CGCCGTGGTGGAGGTGGAGGAGGAGGATGACTCCGAGTCCGAGG-GCTCCATGGTTCCAC
             ||| | ||||||||| |||||||||||| ||| |||||||||| | ||   || ||| |
Sbjct  1391  CGCTGCGGTGGAGGTTGAGGAGGAGGATGGCTCTGAGTCCGAGGAG-TCTGCGGGTCCGC

Query  1729  TTCTGAAGGGTAAACC-CCGGC-CTGCAGGAGGCACGGCTGCCGGAACAGGAACCCCGAC
             |||||||||| ||||| | ||| |||  || ||| |||| || |||  |||| || ||
Sbjct  1450  TTCTGAAGGGCAAACCTCGGGCTCTG--GGTGGCGCGGCGGCTGGAGGAGGAGCCGCG-G

Query  1787  CGGT-GCTCCTGGGACGGCCGCAGGAGGCGTCGCCCTGGTCCCCAAGGAAGATGCCCGCT
             | ||  | || ||| |||| ||||||||||||||||||||||||||||||||| ||||||
Sbjct  1507  CTGTCCCGCCGGGGGCGGCAGCAGGAGGCGTCGCCCTGGTCCCCAAGGAAGATTCCCGCT

Query  1846  TCTCGGCGCCCAGGGGCGCCCTGGCGGAGCACGATGCGTCAG-GGGCGCCCGGGCGCTCC
             |||| |||||||||| |||||||| |||||| || ||| | |  ||||||||||||||||
Sbjct  1567  TCTCAGCGCCCAGGGTCGCCCTGGTGGAGCAGGACGCG-CCGATGGCGCCCGGGCGCTCC

Query  1905  CCTCTGGCCACTACGGTGATGGATTTTATCCACGTGCCCATCCTGCCGCTCAA-CACGGC
             || |||||||| |||||||||||||| |||||||||| |||||||| ||||| ||| ||
Sbjct  1626  CCGCTGGCCACCACGGTGATGGATTTCATCCACGTGCCTATCCTGCCTCTCAATCAC-GC

Query  1964  CTT-TCTGGCCGCCCGCACCCGGCAACTGCTAGAGGGGGATAA-CTATGACGGCGGGGCC
             ||| | |||| |||||||| ||||| ||||| || |  || ||  || ||||||||||||
Sbjct  1685  CTTAT-TGGCAGCCCGCACTCGGCAGCTGCTGGAAGACGA-AAGTTACGACGGCGGGGCC

Query  2022  CCGGCTGTCAGCGCCTTTGCACCGCCGCGGGGCTCGCCCTCG-GCCCCGTCCGCCTCC-G
               ||||| |||||||||||| ||||||||| | || |||| | ||| ||||| || || |
Sbjct  1743  GGGGCTGCCAGCGCCTTTGCCCCGCCGCGGAGTTCACCCT-GTGCCTCGTCCACC-CCGG
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Query  2080  TCACCGCCGGCGACTTCCCCGACTGCGCGTACTCTG-CAGACGACGAGCCCAAGGACGAC
             || | |  ||||||||||||||||||||||| | | | |||| ||||||||||||||||
Sbjct  1801  TCGCTGTAGGCGACTTCCCCGACTGCGCGTAC-CCGCCCGACGCCGAGCCCAAGGACGAC

Query  2139  GCATTCCCGCTCTATGGCGACTTCCAGCCGCCCGCCCTGAAGATCAAGGAAGAGGAGGAA
             || | ||| |||||| |||||||||||||||||| || ||||| ||||| |||||||||
Sbjct  1860  GCGTACCCTCTCTATAGCGACTTCCAGCCGCCCGCTCTAAAGATAAAGGAGGAGGAGGAA

Query  2199  GGCGCCGAGGCCGCCGCGCGCTCTCCGCGGTCGTACCTGGTGGCGGGTGCCAACCCTGCC
             ||||| |||||| |||||||||| ||||| || ||||| ||||| |||||||||| ||
Sbjct  1920  GGCGCGGAGGCCTCCGCGCGCTCCCCGCGTTCCTACCTTGTGGCCGGTGCCAACCCCGCA

Query  2259  GTCTTCCCGGATTTTCCGCT--GG-CGCCGCCGTCGCTGCCTCCGCGAGCG---TCGTCC
             | |||||||||||| ||| |  || | |||||  ||||||| |||||||||    | |||
Sbjct  1980  GCCTTCCCGGATTTCCCGTTGGGGCCACCGCCCCCGCTGCCGCCGCGAGCGACCCCATCC

Query  2313  AGACCTGGGGAAGCCTCGGCGGCGGTCGCACCCACCAGTGCCTCGGTATCGTCGGCGTCC
             ||||| ||||||||  ||| | ||| ||||||| |||||||||| || ||||| ||||||
Sbjct  2040  AGACCCGGGGAAGCGGCGGTGACGGCCGCACCCGCCAGTGCCTCAGTCTCGTCTGCGTCC

Query  2373  TCGTCGGGGTCGGCCTTGGAGTGCATGTTGTATAAGGCGGAGGGCGCGCCGCCCCAACAG
             || ||||||||| || ||||||||||  |||| || |||||||||||||||||||| |||
Sbjct  2100  TCCTCGGGGTCGACCCTGGAGTGCATCCTGTACAAAGCGGAGGGCGCGCCGCCCCAGCAG

Query  2433  GGCCCGTTCCCGCCTCCGCCCTGCAAGCCGCCGAGcgccg-gcgcctgcctgctgccgcg
             ||||||||| |||| |||||||||||| ||||| ||| || ||| ||||||||| |||||
Sbjct  2160  GGCCCGTTCGCGCCGCCGCCCTGCAAGGCGCCGGGCG-CGAGCGGCTGCCTGCTCCCGCG

Query  2492  ggacagcctgccgtccacctcggcctccgccgccgccgccgccgccgccgccgccgGGGC
             |||| ||||||| |||||||| ||||   | |||||||||||||  |  |  | |  |||
Sbjct  2219  GGACGGCCTGCCCTCCACCTCCGCCT---CTGCCGCCGCCGCCG--G--G--G-C--GGC

Query  2552  GGCTCCTGCGCTCTA-CCAGCCGCTTGGCCTTAACGGGCTCCCGCAGCTCGGCTACCAGG
               | || |||||||| || | | || ||||| ||||||||||||||||||||||||||||
Sbjct  2267  --C-CCCGCGCTCTACCCTG-CACTCGGCCTCAACGGGCTCCCGCAGCTCGGCTACCAGG

Query  2611  CCGCGGTACTCAAGGAGGGCCTGCCGCAGGTGTACCAGCCCTATCTCAACTACCTGAGGC
             |||| || |||||||||||||||||||||| |||| ||||||||||||||||||||||||
Sbjct  2323  CCGCCGTGCTCAAGGAGGGCCTGCCGCAGGTCTACCCGCCCTATCTCAACTACCTGAGGC

Query  2671  CGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTCGAGTCATTACCTCAGAAGATTT
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  2383  CGGATTCAGAAGCCAGCCAGAGCCCACAATACAGCTTCGAGTCATTACCTCAGAAGATTT

Query  2731  GTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGGAGCT
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2443  GTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTGTCCTTACCTGTGGGAGCT

Query  2791  GTAAGGTCTTCTTTAAAAGGGCAATGGAAGGGCAGCACAACTACTTATGTGCTGGAAGAA
             |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  2503  GTAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCACAACTACTTATGTGCTGGAAGAA

Query  2851  ACGACTGCATTGTTGATAAAATCCGCAGAAAAAACTGCCCAGCTTGTCGCCTTAGAAAGT
             | |||||||| |||||||||||||||||||||||||||| ||||||||||||||||||
Sbjct  2563  ATGACTGCATCGTTGATAAAATCCGCAGAAAAAACTGCCCAGCATGTCGCCTTAGAAAGT

Query  2911  GCTGTCAGGCTGGCATGGTCCTTGGAGGTCGAAAGTTTAAAAAGTTCAATAAAGTTAGAG
             |||||||||||||||||||||||||||||||| |||||||||||||||||||| ||||
Sbjct  2623  GCTGTCAGGCTGGCATGGTCCTTGGAGGTCGAAAATTTAAAAAGTTCAATAAAGTCAGAG
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Query  2971  TTATGAGAGCACTAGATGCCGTTGCTCTCCCACAGTCGGGGGGCATTCCAAATGAAAGTC
             || |||||||||| ||||| |||||||||||||| | | |||| |||||||||||||| |
Sbjct  2683  TTGTGAGAGCACTGGATGCTGTTGCTCTCCCACAGCCAGTGGGCGTTCCAAATGAAAGCC

Query  3031  AGGCCCTAAGCCAGAGAATCACTTTTTCACCAAATCAAGACTTACAGCTGATCCCCCCGT
             | ||||||||||||||| |||||||||||||  ||||||| ||||| |||| || ||
Sbjct  2743  AAGCCCTAAGCCAGAGATTCACTTTTTCACCAGGTCAAGACATACAGTTGATTCCACCAC

Query  3091  TGATCAACTTGCTCATGAGCATTGAACCAGATGTGGTCTATGCAGGACATGACAACACCA
             |||||||| || | |||||||||||||||||||| |||||||||||||||||||||| |
Sbjct  2803  TGATCAACCTGTTAATGAGCATTGAACCAGATGTGATCTATGCAGGACATGACAACACAA

Query  3151  AACCCGACACTTCGAGTTCTTTGCTGACAAGTCTGAATCAGCTGGGTGAAAGACAACTTC
             |||| ||||| || |||||||||||||||||||| ||||| || || || || |||||||
Sbjct  2863  AACCTGACACCTCCAGTTCTTTGCTGACAAGTCTTAATCAACTAGGCGAGAGGCAACTTC

Query  3211  TTTCAGTAGTCAAGTGGTCTAAGTCACTGCCAGGTTTTCGAAACTTACATATTGATGACC
             |||||||||||||||||||| ||| ||||||||||||||||||||||||||||||||||
Sbjct  2923  TTTCAGTAGTCAAGTGGTCTAAATCATTGCCAGGTTTTCGAAACTTACATATTGATGACC

Query  3271  AAATAACTCTCATCCAGTATTCCTGGATGAGCTTAATGGTATTTGGACTAGGATGGAGAT
             | |||||||||| |||||||| |||||||||||||||| ||||| ||||||||||||
Sbjct  2983  AGATAACTCTCATTCAGTATTCTTGGATGAGCTTAATGGTGTTTGGTCTAGGATGGAGAT

Query  3331  CCTATAAACATGTCAGTGGACAGATGCTTTATTTTGCACCTGATCTAATACTAAATGAAC
             |||| ||||| |||||||| |||||||| ||||||||||||||||||||||||||||||
Sbjct  3043  CCTACAAACACGTCAGTGGGCAGATGCTGTATTTTGCACCTGATCTAATACTAAATGAAC

Query  3391  AGCGGATGAAGGAATCATCATTCTATTCATTATGCCTTACCATGTGGCAGATCCCACAGG
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3103  AGCGGATGAAAGAATCATCATTCTATTCATTATGCCTTACCATGTGGCAGATCCCACAGG

Query  3451  AGTTTGTGAAGCTTCAAGTTAGCCAAGAAGAGTTTCTCTGTATGAAAGTATTGCTACTTC
             ||||||| |||||||||||||||||||||| |||||||||||||||| ||||||
Sbjct  3163  AGTTTGTCAAGCTTCAAGTTAGCCAAGAAGAGTTCCTCTGTATGAAAGTATTGTTACTTC

Query  3511  TTAATACAATTCCTTTGGAAGGACTAAGAAGTCAAAACCAGTTTGAAGAGATGAGATCAA
             |||||||||||||||||||| ||| ||||||||| ||||||||| |||||||| ||||
Sbjct  3223  TTAATACAATTCCTTTGGAAGGGCTACGAAGTCAAACCCAGTTTGAGGAGATGAGGTCAA

Query  3571  GCTACATTAGAGAGCTCATCAAGGCAATTGGTTTGAGACAAAAAGGAGTCGTCCCTAGCT
             |||||||||||||||||||||||||||||||||||| |||||||||| ||  | ||||
Sbjct  3283  GCTACATTAGAGAGCTCATCAAGGCAATTGGTTTGAGGCAAAAAGGAGTTGTGTCGAGCT

Query  3631  CACAGCGTTTCTACCAGCTTACAAAACTTCTTGATAACTTGCATGATCTTGTCAAACAAC
             |||||||||||| || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  3343  CACAGCGTTTCTATCAACTTACAAAACTTCTTGATAACTTGCATGATCTTGTCAAACAAC

Query  3691  TTCATCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAATTTCCAG
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3403  TTCATCTGTACTGCTTGAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAATTTCCAG

Query  3751  AAATGATGTCTGAGGTTATTGCTGCACAATTACCCAAGATCTTGGCAGGGATGGTGAAAC
             |||||||||||| |||||||||||||||||||| ||||||||||||||||||||
Sbjct  3463  AAATGATGTCTGAAGTTATTGCTGCACAATTACCCAAGATATTGGCAGGGATGGTGAAAC

Query  3811  CCCTTCTCTTTCATAAAAAGTGAATGCCAT-TTTTATCTTTAAAA-AATTAAACTTTGTG
             |||||||||||||||||||||||||| ||| |||| ||||| ||| ||||||| ||||||
Sbjct  3523  CCCTTCTCTTTCATAAAAAGTGAATGTCATCTTTT-TCTTTTAAAGAATTAAATTTTGTG
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Query  3869  GTATATCTGTTTTCGTTTTTGTCAGGATTATGAGGTCTTGAGTTTTTATAATGTTCT-C-
             |||| ||| |||| ||||| |||||||||||||||||||||||||||||||||||| |
Sbjct  3582  GTATGTCT-TTTT-GTTTTGGTCAGGATTATGAGGTCTTGAGTTTTTATAATGTTCTTCT

Query  3927  -AAAGCCCTACATTTATAACAT-ATTGTACTGTGTAAATTTCAGAGGAAAAAT-GTGAGG
              |||||| |||||||||||||| || ||   |||||||||| | || |||||| ||||||
Sbjct  3640  GAAAGCCTTACATTTATAACATCATAGT---GTGTAAATTTAAAAG-AAAAATTGTGAGG

Query  3984  GTG-AATTGGATTTGCTTT-ATAAAGCATTATTTGAATTTTTAG-TGTTTTTGCGTACCC
               |  ||||  |||| |||| |||||| || ||| |||| ||||  |||||| |  |||||
Sbjct  3696  TTCTAATT--ATTTTCTTTTATAAAGTATAATTAGAATGTTTAACTGTTTT-GTTTACCC

Query  4041  ATATTTTCTTTAAGAGTTTACACGATTGAAAAAGTACTAAGTTGATTGTTAAAGTTAA-T
             |||||||||| |||| |||||| ||||||||||||||||    |||||||||| || |
Sbjct  3753  ATATTTTCTTGAAGAATTTACAAGATTGAAAAAGTACTAAA---ATTGTTAAAGTAAACT

Query  4100  C-CT-ATCCGTATCATTTCATACCATTTAGGTGAG-ATTTTTAACTTTCACAGCTAACAG
                || |||| ||| |||||||||||| |||||||| ||||||||||||  || ||||||
Sbjct  3810  ATCTTATCCATATTATTTCATACCATGTAGGTGAGGATTTTTAACTTTTGCATCTAACAA

Query  4157  ATCCTCTACTTTAGAGGaaaaaaatcttacacataaaaataaaaaGCTATTATATATGTT
             ||| || |||| |||| |||||| |||||||  ||| || | ||||||||||||  |||
Sbjct  3870  ATCATCGACTTAAGAG-AAAAAA-TCTTACATGTAATAACACAAAGCTATTATAT--GTT

Query  4217  CCTTCTATGTAGCTCCCTTTGTCTCCCTGATTATATTTACAAAACTGAAACTTTAAAATG
               ||||| ||| |||||||||| ||    ||||||||| ||||| |||| ||||||||||
Sbjct  3926  ATTTCTAGGTAACTCCCTTTGTGTCA---ATTATATTTCCAAAAATGAACCTTTAAAATG

Query  4277  GTATGCAAAACTT-GTCTATATGAGTGTACATACAttttttGTGAGAAGGATATTTATA
             |||||||||| || |||||||| | |  |  |   || |   ||||| |||| ||| |||
Sbjct  3983  GTATGCAAAATTTTGTCTATAT-A-T--A--T---TTGT---GTGAGGAGGAAATTCATA

Query  4336  ATCTTAA-TCAGTTTTTCCAAAGGGTTTTTAATGCAAAAATATATATATACAGAAAAAGA
             | |||   |||| ||||| ||||  |||||||||||||| || ||      ||||  ||
Sbjct  4031  A-CTTTCCTCAGATTTTCAAAAGTATTTTTAATGCAAAAA-ATGTA------GAAA--GA

Query  4395  GTT-AACAATT-CTTTAATTAGAT-GATACTCCTCAAACTAGG-AAAACCAGCTTATATG
             ||| || ||   ||  ||| |||| |||  || |||||||||| ||||| | || |||||
Sbjct  4081  GTTTAA-AACCACTAAAAT-AGATTGATGTTCTTCAAACTAGGCAAAACAA-CTCATATG

Query  4451  TTAAGACTGTTTCCCAGATTGGAAACACAAATCTCT-AGGGAAGTTATTAAGTAGA  4505
             |||||||  ||| |||||||||||||||||||||| ||| |||||| ||||||||
Sbjct  4138  TTAAGACCATTTTCCAGATTGGAAACACAAATCTCTTAGG-AAGTTAATAAGTAGA  4192
```

Sequences were also compared using ClustalW2 software. Porcine PGR mRNA had 79% identity to human PGR sequence, 63% identity to the mouse PGR sequence and 84% identity to bovine PGR sequence (Table 3.2). Low sequence coverage of the 5' upstream region of porcine PGR in the human, mouse and cow mRNA sequences, and homology is low until the PGR-B translation start codon at 1029 bp. Homology is moderate from 1029 – 2532 bp located in exon 1 and encoding the variable A/B domain of the PGR protein based on human identification of domains. Homology is high from 2629 – 3379 beginning in the 3' region of exon 1 and terminating around the end of exon 5. Homology is also high from 3520 – 3833 bp which correlates to the beginning of exon 7 through exon 8, including the stop codon (Figure 3.8).

The NCBI ORF Finder program identified 3 possible open reading frames in the porcine PGR nucleotide sequence. As anticipated, these 3 start codons are in-frame with one another and result in 3 putative proteins that differ only in N-terminus truncations (Figure 3.9). The porcine PGR transcript was translated into a putative full-length PGR protein that was 934 amino acids in length (NP001159960.1; Figure 3.10). Homology between mRNA derived protein sequence and genomic DNA derived protein sequence conserved 98% identity (918/934 amino acids with no gaps) using the BLASTP program (Figure 3.11). Fifteen residues were not identical and with no gaps in the alignment. Of those 15 amino acid differences, 11 had no similarity in amino acid residue stoichiometry and 4 had similarly charged amino acid residue substitutions.

The overall homology to human PGR protein observed 84% identity (NP000917.1) when aligned using ClustalW2. The porcine PGR protein was 75% identical to human PGR protein within the A/B (variable) region and was 97% identical

Table 3.2 Matrix of nucleotide identity (%) between mouse (NP032855.2), predicted pig, human (NP000917.3), and predicted cow (XP583951.5) PGR protein from the first methionine codon (PGR-B). Alignment was generated and identity percentage calculated by ClustalW2 software.

	Mouse	**Pig**	**Human**	**Cow**
Mouse		63	42	76
Pig	63		79	84
Human	42	79		82
Cow	76	84	82	

Figure 3.8 Nucleotide multiple sequence alignment of pig (**P**; GQ903679.1), human (**H**; NM000926.4), cow (**C**; XM583951.5), and mouse (**M**; NM00829.2) PGR mRNA from the first nucleotide residue of porcine sequenced transcript. Alignment generated by ClustalW2 software. Stars (*) indicate residues identical in all 4 species. The translation start codons (ATG) are shown in bold, underlined text: PGR-B at base 1029 (reference sequence is P: GQ903679.1); PGR-A at base 1516; PGR-C at base 2815. The stop codon (TGA) located at base 3829 is shown in italics, double underlined text. Low sequence coverage in other species in the 5' upstream region of porcine PGR and homology is low until the PGR-B translation start codon at 1029 bp. Homology is moderate from 1029 – 2532 bp. Homology is high from 2629 – 3379 and 3520 – 3833, including the stop codon sequence.

```
P      TTCTTCTTCCAGGGAAAAATCTTTACAAAATTGAAATCTTGAAAATTCAGCTTCTGATAG 60
H      ------------------------------------------------------------
C      ------------------------------------------------------------
M      ------------------------------------------------------------

P      AATCCATTCGCTTTTCCAGTTAGACTGGGCATAATTCTTAATTGGTAGTACACAATCTAA 120
H      ------------------------------------------------------------
C      ------------------------------------------------------------
M      ------------------------------------------------------------

P      TATGCTTACGAAGTTCCATCCTCACAGTACCTGTTTTTTAGACTAGCGGTTGGGAATACT 180
H      ------------------------------------------------------------
C      ------------------------------------------------------------
M      ------------------------------------------------------------

P      AACTCCAGAATTTCAGATCTGGTCCATAATTGGGGCAGGGAGGGGCTTTGGGCGGAGCCT 240
H      ------------------------------------------------------------
C      ------------------------------------------------------------
M      ------------------------------------------------------------

P      CCGGATAGGGAGGAGGCATTGTTAGAAAGCTGTGGGGCTAGTCTACTGGCTGTCACTACT 300
H      ------------------------------------AGTCCAC-AGCTGTCACTAAT 20
C      ------------------------------------------------------------
M      ----------------------------------CAGTCTA--GACTGTCACTA-- 18

P      CTAGTTAAGCCTTTGTATTTGAATGTGCGAGGAGGGTCTGCTGTGTTAGCAGCATTTTCA 360
H      CGGGGTAAGCCTT----GTTGTATTTGTG-----------CGTGTGGGTGGCATTCTCA 64
C      ------------------------------------------------------------
M      -----TCAGTCTTT---GTAGTATTTACG--------------GGTGCGAGGATTCTGA 55

P      GTGAGAACTAGTCTCACTTGGCATT-TGAGTGGAATCTGCACCTACAGGCGTTTCGTCCC 419
H      ATGAGAACTAGCTTCACTTGTCATT-TGAGTGAAATCTACAACCCGAGGCGGCTAGTGCT 123
C      ------------------------------------------------------------
M      CATTCTGCTAACTTCTCTTAGCTTTACTAGAGCAACCTGCAACCAGAACTCGCTGG---- 111

P      CTCCGGACTGCTAAGAAATGTGAGGGCTGCGGAGATGACTGTCGCCAGCTATAGGGAACC 479
H      C-CCGCACTACTGGGA--TCTGAGATCTTCGGAGATGACTGTCGCCCGCAGTACGGAGCC 180
C      ------------------------------------------------------------
M      -----GATTGTAGAGAACCTAGGGAGCCATAGAGA---CTGTCGCT-------------- 149

P      GGGAGGTATAAGACTGTTCA-GGGAATGGGCTGTGCCGAGAGGCCAAAATAGCCCCAGGG 538
H      AGCAGAAGTCCGACCCTTCCTGGGAATGGGCTGTACCGAGAGGTCCGACTAGCCCCAGGG 240
C      ------------------------------------------------------------
M      -GCAGGAGAACGAGT------AAGAATGGGCTACGTC-----------TTAGCCCCAGGT 191
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P      TTTTAGGGAGACGGGAAATGTAACACAGGCAGGGACTGGCAGCAACAGGGCAT------- 591
H      TTTTAGTGAGG-GGGCAGTGGAACTCAGCGAGGGACTGAGAGCTTCACAGCATGCACGAG 299
C      ------------------------------------------------------------
M      TTTTGGTGAGGACTGTAAAGCAAACTTCCTAGGATGTCTAGGTTTCAACAAAC------- 244

P      --TGAAGCCGGAGAAAAAGTCGGGAGATA---GAGCCGCCAGT-----AATTGCTTTCGA 641
H      TTTGATGCCAGAGAAAAAGTCGGGAGATAAAGGAGCCGCGTGTCACTAAATTGCCGTCGC 359
C      ------------------------------------------------------------
M      ----------ATAAGAAGTCCGGAGATAG------CGGGAGT-------CCTTTTTTTC 280

P      AGCCGCCGCCACTCGAGTGCTG-ACTTGTGAGTACTCTGCGTCTCCTGTCTTCAGACTGA 700
H      AGCCGCAGCCACTCAAGTGCCGGACTTGTGAGTACTCTGCGTCTCCAGTCCTCGGACAGA 419
C      ------------------------------------------------------------
M      AGCACCGGCCACACCAGT----------------TCCGGGTCTCCCATTCAAGAAGAAA 323

P      -AGTCGGGGAACTCTCTTGGAGAACTCTCCCAGTTAGGAAACGAGATCCCT-----ACAA 754
H      -AGTTGGAGAACTCTCTTGGAGAACTCCCCGAGTTAGGAGACGAGATCTCCTAACAATTA 478
C      ------------------------------------------------------------
M      CACGAAAAAAAGTCTCTCGAATAACTCCC--AGTTCTCAGACCAGACCAGCTTGCTCCAG 381

P      CTACCTTCGCCGGCTCTCCCCACCTGCCTCA----AGGACGTAACAACTATCACCCATCT 810
H      CTACTTTTTCTTGCGCTCCCCACTTGCCGCTCGCTGGGACA-AACGACAGCCACAGTTCC 537
C      ------------------------------------------------------------
M      CTACTTCTTCCTGTCCTCACC--CCACCGCG-ACCGGGACAGCGCGACTACCACCCTTCC 438

P      CCTGCCTACCAGACGCTGGGCAAGGGCAGGAGCTGACAGGTGCCGCCCCCTTCCGACCCA 870
H      CCTGACGACAGGATGGAGGCCAAGGGCAGGAGCTGACCAGCGCCGCCCTCCCCCGCCCCC 597
C      ------------------------------------------------------------
M      TCTG-CGTCTGGGTGGAGGGTAAGGACAGGAGCTGACCAAGACCGCCCCTCCC------- 490

P      GGAGGTGGAGATTTCACCCCCCTCTCCACCGTCCCGTCCAGCCAAATTCAACACCTATTT 930
H      GACCCAGGAGGTGGAGATCCC----------TCCGGTCCAGCCACATTCAACACCCACTT 647
C      ------------------------------------------------------------
M      -AACCAGGAGGTGGAGATCCC----------ACGGGTCCTGCGAC--TCTACACCCACTT 537

P      TCTCCTCCCGCTGCCCCTATATTCCCGACACCCCCTCATCCTTCCCCTATCCCTCCTCCC 990
H      TCTCCTCCCTCTGCCCCTATATTCCCGAAACCCCCTCCTCCTTCCCTTTTCCCTCCTCCT 707
C      ------------------------------------------------------------
M      CCTCCTCCCTCTGCCCCTATATCACCGGCACCCCCTCCTCCTTCCCTTTTCCCTCCTCCC 597

P      CAGAGACAGGGGAGGAGAAAAGGGGAGTCCAGGTCGTC<u>ATG</u>ACTGAGCTGAAGGCAAAGG 1050
H      -GGAGACGGGGGAGGAGAAAAGGGGAGTCCAG-TCGTC<u>ATG</u>ACTGAGCTGAAGGCAAAGG 765
C      ------------------------------------<u>ATG</u>ACTGAGCTGAAGGCGAAGG 22
M      AGGAGACAGGGGAGGAGAAAAGGGGAGCTTGGGTCGTC<u>ATG</u>ACTGAGCTGCAGGCAAAGG 657
                                             ************ **** ****
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P      ATCCCCGGGCTCCCCACGTGGCGGGCAGAGCGCCCTCCCCCA------CCCAGCTCGGGA 1104
H      GTCCCCGGGCTCCCCACGTGGCGGGCGGCCCGCCCTCCCCCGA---GGTCGGATCCCCAC 822
C      GTCCCCGCGCTCCCCACGTGGCGGGCACAGCGCCCTCGCCCACCCAGGCCGGAGCCCCTC 82
M      ATCCGCAGGTTCTCCACACGTCTGGCGCTTCGCCCTCCCCCCCACACATCGGGTCCCCCT 717
        *** *  * ** ****  * * ***    ******* ***        *     *

P      CGCTGGGACGCCCAGACACAGGCCCCTTTCAGGCGAGCCAGACCTCGGAAGCGTCGCCCG 1164
H      TGCTGTGTCGCCCAGCCGCAGGTCCGTTCCCGGGGAGCCAGACCTCGGACACCTTGCCTG 882
C      TGCGGGGACGCCCAGACGCGGGCCCCTTCCAGGCGGGCGAGGCCTCGGGCCCGACGCCCA 142
M      TGCTTGCACGCTTGGACTCAGGTCCCTTCCAAGGGAGCCAGCACTCGGACGTGTCGTCTG 777
        **     ***   * * * ** ** ** *  * * ** **  *****       * *

P      CAGCCTCGGCCATACCCCTCTCCCTGGACGGGCTACTCTTCCCTGGGCCCTGCCAGGGAC 1224
H      AAGTTTCGGCCATACCTATCTCCCTGGACGGGCTACTCTTCCCTCGGCCCTGCCAGGGAC 942
C      CAGCCTCGGGCCTCCCCCTCTCCCTGGACGGACTGATCTTCCCTCGGTCCTGCCAGGCTC 202
M      TAGTCTCGCCTATACCGATCTCCCTGGACGGGCTGCTTTTTCCTCGGTCCTGCCGGGGTC 837
        **  ***    * **  ************ **  * ** *** ** ****** **  *

P      AGGAACCA---GACGGGAAGACGCAGGACCAGCAGTCGCTGTCAGACGTGGAGGGGGCGT 1281
H      AGGACCCCTCCGACGAAAAGACGCAGGACCAGCAGTCGCTGTCGGACGTGGAGGGCGCAT 1002
C      AGGACCTG---GACGGGAAGACGCCGGACCCAGAGTCGCTGGCAGACGTGGAGGGGGCGT 259
M      CCGAGCTCCCAGACGGAAAGACAGGGGACCAGCAGTCGCTGTCCGACGTGGAGGGAGCTT 897
         ** *     ****  *****   *****   ******** * ********** ** *

P      ATCCCACAGTTGAAGCTACAGAGGGTGCTGGAGGTGGCAGCTCTAGACCCTCGGAAAAAG 1341
H      ATTCCAGAGCTGAAGCTACAAGGGGTGCTGGAGGCAGCAGTTCTAGTCCCCCAGAAAAGG 1062
C      ATTCCGGAGGGGAAGCCGCAGAGGGCGCTGCTG---------CGAGACCCCCAGAGAAGG 310
M      TCTCTGGGGTAGAAGCCACTCATAGGGAAGGAGGCAGAAATTCCAGAGCCCCGGAGAAGG 957
          *    *  *****  *     * *  *  *          * **  ** * ** ** *

P      ACACCGGGCTGCTGGACAGTGTCTTGGACACGCTACTAGCGCCCTCAGGTCCCGGGCAGA 1401
H      ACAGCGGACTGCTGGACAGTGTCTTGGACACTCTGTTGGCGCCCTCAGGTCCCGGGCAGA 1122
C      ACGGCGGGTTGCTGGACAGCGTCCTGGACACCCTGCTGGCGCCCCCGGGCCCTGAGCGGA 370
M      ACAGCAGACTCTTAGACAGTGTCTTAGACTCGTTGTTGACTCCCTCCGGGACCGAACAGA 1017
       **  * *  *  * ***** *** * *** *  *  *  * *** * **  * *  * **

P      GCCACGCCAGCCCTCCCGCCTGCGAAGCCACCAGCCCTTGGTGCTTGTTTGGCTCTGAGC 1461
H      GCCAACCCAGCCCTCCCGCCTGCGAGGTCACCAGCTCTTGGTGCCTGTTTGGCCCCGAAC 1182
C      GCCCCGCCAGCCCCGCCGTCTGCGAAGCCCCCAGCCCTTGGTGCCTCTTTGGCCCCGAGC 430
M      GTCACGCCAGCCCTCCAGCCTGCGAGGCCATCACTTCCTGGTGTCTCTTTGGGCCAGAGC 1077
       * *   *******  * * ****** * *  **   * *****  * *****  * ** *

P      TTCCCCAGGACGCTCGGGTTGCCCCTTCCACCCAGGGAGTATTGCCCCTGCTC<u>**ATG**</u>AGCC 1521
H      TTCCCGAAGATCCACCGGCTGCCCCCGCCACCCAGCGGGTGTTGTCCCCGCTC<u>**ATG**</u>AGCC 1242
C      TTGTCCAAGACGCCCGGGCTGCGCCAGCCGCCCAGGGGGTCCTACTCCCACTC<u>**ATG**</u>AGCC 490
M      TTCCAGAAGACCCCCGCAGTGTCCCTGCTACCAAAGGGTTGTTGTCCCCGCTC<u>**ATG**</u>AGTC 1137
       **    * **  * *    **  **  *  ** *  *  *  *   **  ******** *

P      GGCCAGAGGGCAAGGCTGGCGACAGCTCCGGGACGGCAGCCGCCCATAAAGTGCTATCCA 1581
H      GGTCCGGGTGCAAGGTTGGAGACAGCTCCGGGACGGCAGCTGCCCATAAAGTGCTGCCCC 1302
C      GCCCAGAGAGCAAGGCTGGCGACACCCCCGGGGCCGCCGCCGGCCAGAAGGTGCTGCCCC 550
M      GGCCAGAGATCAAGGCTGGCGACAGCTCCGGGACAGGAGCAGGACAGAAGGTGCTGCCCA 1197
       *  * * *  ***** *** **** * ***** * *  ** *  ** ** *****  **
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```
P     GGGGTCTGGCACCGTCCCGGCAGCTGCTGCCCTCGACTGCCGGGAGCCATCACTGGCCGG 1641
H     GGGGCCTGTCACCAGCCCGGCAGCTGCTGCTCCCGGCCTCTGAGAGCCCTCACTGGTCCG 1362
C     GGGGCCTGGCGCCATGCCGGCAGCTGCTGGCCCCGACGGCCTCGAGCCACCCCTGGCCCG 610
M     AAGGACTGTCACCACCCAGGCAGCTGTTGCTCCCTACCTCGGGGAGTGCTCACTGGCCCG 1257
        ** *** * **   * ******** **  * *  *  *   ***    * **** * *

P     CGGCCGCAGTGAAGCCCTCTCCGCAGCCCGCCGTGGTGGAGGTGGAGGAGGAGGATGACT 1701
H     GGGCCCCAGTGAAGCCGTCTCCGCAGGCCGCTGCGGTGGAGGTTGAGGAGGAGGATGGCT 1422
C     CGCCCGCGCCGAGGCCGTCTCCGCAGCCCGCGGTGGTGGAGCT---GGAGGAGGACGACT 667
M     GGGCAGGGGTGAAGCCGTCCCCGCAGCCAGCTGCAGGGGAGGTGGAAGAGGACAGTGGCC 1317
       * *      ** *** ** ****** * ** *  * **** *    *****    * *

P     CCGAGTCCGAGGGCTCCATGGTTCCACTTCTGAAGGGTAAACCCCGGCCTGCAGGAGGCA 1761
H     CTGAGTCCGAGGAGTCTGCGGGTCCGCTTCTGAAGGGCAAACCTCGGGCTCTGGGTGGCG 1482
C     CGGATCCCGAGGGCTCCTCGGGGCCGCCCCTGAAGGGCAAAGCCCGGCCCGCGGG----- 722
M     TGGAGACCGAGGGCTCTGCAGCTCCGCTTCTAAAGAGCAAACCTCGAGCACTGGAAGGCA 1377
        **  ******  **    *  ** *  ** *** * *** * **  *    *

P     CGGCTGCCGGAACAGGAACCCCGACCGGTGCTCCTGGGACGGCCGCAGGAGGCGTCGCCC 1821
H     CGGCGGCTGGAGGAGGAGCCGCGGCTGTCCCGCCGGGGGCGGCAGCAGGAGGCGTCGCCC 1542
C     -------------AGGAGACGCGATCAGCGCTCCTGGGACGGCCGCGGGAGGCGTCGCCC 769
M     CCGGCAGCGGAGGAGGAGTCGCAGCCAACGCGGCCTCAGCGGCCCCAGGCGGTGTCACTC 1437
                 ****  * *        *  *     ****  * ** ** *** * *

P     TGGTCCCCAAGGAAGATGCCCGCTTCTCGGCGCCCAGGGGCGCCCTGGCGGAGCACGATG 1881
H     TGGTCCCCAAGGAAGATTCCCGCTTCTCAGCGCCCAGGGTCGCCCTGGTGGAGCAGGACG 1602
C     CGGTGCCCAAAGAAGATGCCCGCTTCTCGGCGCCCAGGGCCGCCCCGGCGGAGCATGACG 829
M     TGGTCCCAAAGGAAGATTCACGGTTTTCTGCTCCTAGGGTCTCCTTGG---AGCAAGACT 1494
       *** ** ** ****** * ** ** ** ** ** **** * **  **   **** **

P     CGTCAGGGGCGCCCGGGCGCTCCCCTCTGGCCACTACGGTGATGGATTTTATCCACGTGC 1941
H     CGCCGATGGCGCCCGGGCGCTCCCCGCTGGCCACCACGGTGATGGATTTCATCCACGTGC 1662
C     CACCCGCCGCGCCCGGGCGCGCCTCGGTGGCCACCGCGGTGATGGACTTCATCCACGTGC 889
M     CTCCCATTGCCCCCGGGCGCTCCCCACTGGCCACCACAGTGGTGGATTTCATCCATGTGC 1554
      *  *    ** ********* ** *  *******  * *** **** ** ***** ****

P     CCATCCTGCCGCTCAACACGGCCTTTCTGGCCGCCCGCACCCGGCAACTGCTAGAGGGGG 2001
H     CTATCCTGCCTCTCAATCACGCCTTATTGGCAGCCCGCACTCGGCAGCTGCTGGAAGACG 1722
C     CCATCCTGCCGCTCAACTCAGCCTTCCTGGCCGCCCGCACCCGGCAGCTGCTGGAGGGTG 949
M     CCATCCTGCCTCTGAACCACGCACTCCTGGCCGCCCGCACCCGGCAGCTGCTGGAGGGGG 1614
      * ******** ** **    **  *  **** ******** ***** ***** ** *  *

P     ATAACTATGACGGCGGGGCCCCGGCTGTCAGCGCCTTTGCACCGCCGCGGGGCTCGCCCT 2061
H     AAAGTTACGACGGCGGGGCCGGGGCTGCCAGCGCCTTTGCCCCGCCGCGGAGTTCACCCT 1782
C     ACAGCTATGACGGCGGGGCC------GCCGGCGCCTTCGCCCCGCCGCGGGGCTCGCCCT 1003
M     ACAGCTACGACGGCGGGGCCACAGCCCAGGGCCCTTTTGCCCCGCCTAGGGGCTCGCCCT 1674
      * *  ** ************          ** * ** ** *****  ** * ** ****

P     CGGCCCCGTCCGCCTCCGTCACCGCCGGCGACTTCCCCGACTGCGCGTACTCTGCAGACG 2121
H     GTGCCTCGTCCACCCCGGTCGCTGTAGGCGACTTCCCCGACTGCGCGTACCCGCCCGACG 1842
C     CCGCCTCGGCCGCCCTGGGCGCGCCCGGAGACTTCCCCGACTGCGCGTACCAGCCCGACT 1063
M     CCGCGCCATCCCCGCCGGTGCCCTGCGGTGACTTCCCAGACTGCACCTACCCTCTGGAAG 1734
        **  *  ** *    *   *    ** ******** ****** * ***      **
```

```
P      ACGAGCCCAAGGACGACGCATTCCCGCTCTATGGCGACTTCCAGCCGCCCGCCCTGAAGA 2181
H      CCGAGCCCAAGGACGACGCGTACCCTCTCTATAGCGACTTCCAGCCGCCCGCTCTAAAGA 1902
C      CCGAGCCCAAGGACGACGCGTTCTCGCTCTACGGGGACCCGCAGCCGCCCGCCCTGAAGA 1123
M      GCGACCCCAAAGAGGACGTGTTCCCTCTTTACGGCGACTTCCAGACGCCTGGCTTGAAGA 1794
        *** ***** ** ****  * * * ** **  * ***   *** **** *   * ****

P      TCAAGGAAGAGGAGGAAGG---CGCCGAGGCCGCCGCGCGCTCTCCGCGGTCGTACCTGG 2238
H      TAAAGGAGGAGGAGGAAGG---CGCGGAGGCCTCCGCGCGCTCCCCGCGTTCCTACCTTG 1959
C      TCAAGGAGGAGGAGGAGGGGGGCGCCGAGGCAGCCGCGCGCTCCCCGCGGCCGTACCTGC 1183
M      TCAAGGAGGAGGAGGAGGG---CGCGGATGCTGCTGTGCGCTCGCCGCGCCCCTACCTGT 1851
       * ***** ******** **   *** ** **  * * ****** *****  * *****

P      TGGCGGGTGCCAACCCTGCCGTCTTCCCGGATTTTCCGCTGGCGCCGCCGTC---GCTGC 2295
H      TGGCCGGTGCCAACCCCGCAGCCTTCCCGGATTTCCCGTTGGGGCCACCGCCCCCGCTGC 2019
C      TGGCGGGGGCCAGCCCCGCCGTCTTCGCCGACTTCCCGCTGGCGCCGCCGTC-------- 1235
M      CGGCTGGAGCCAGCTCCTCCACCTTCCCAGACTTCCCGCTGGCACCCGCGCC-------- 1903
        *** ** **** * *  *   **** * ** ** *** ***  **  ** *

P      CTCCGCGAGCG---TCGTCCAGACCTGGGGAAGCCTCGGCGGCGGTCGCACCCACCAGTG 2352
H      CGCCGCGAGCGACCCCATCCAGACCCGGGGAAGCGGCGGTGACGGCCGCACCCGCCAGTG 2079
C      ------------------CAGGCCGGGGGAGGTGGCGGTGCCCGCGGCCGCCACGGGGG 1276
M      -GCAGCGAGCGCCATCCTCCAGGCCCGGAGAAGCGGCGGTGGCCGGCGGACCCAGCAGCG 1962
                       *** ** ** ** *   *** * * *  *   **    * *

P      CCTCGGTATCGTCGGCGTCCTCGTCGGGGTCGGCCTTGGAGTGCATGTTGTATAAGGCGG 2412
H      CCTCAGTCTCGTCTGCGTCCTCCTCGGGGTCGACCCTGGAGTGCATCCTGTACAAAGCGG 2139
C      CCCCCGGCTCGTCCGCGGCCGTGTCGGGGTCAGCCCTGGAGTGCGTCCTGTACAAGGCGG 1336
M      CCGCGGTGTCGCCAGCGTCCTCCTCCGGCTCCGCGCTGGAGTGCATCCTGTACAAAGCGG 2022
       ** * *  *** * *** **   ** ** **  *  ******** *  **** ** ****

P      AGGGCGCGCCGCCCCAACAGGGCCCGTTCCCGCCTCCGCCCTGCAAGCCGCCGAGCGCCG 2472
H      AGGGCGCGCCGCCCCAGCAGGGCCCGTTCGCGCCGCCGCCCTGCAAGGCGCCGGGCGCGA 2199
C      AGGGCGCGCCGCCCCAGCCGGGCCCGTTCGCGCCGCCGCCCTGCAAGGCTCCGGCCGCAG 1396
M      AGGGCGCGCCGCCCACGCAGGGTTCGTTCGCGCCACTGCCGTGCAAGCCCCCAGCCGCCG 2082
       **************   * ***  ***** **** * *** ****** * **   ***

P      GCGCCTGCCTGCTGCCGCGGGACAGCCTGCCGTCCACCTCGGCCTCCGCCGCCGCCGCCG 2532
H      GCGGCTGCCTGCTCCCGCGGGACGGCCTGCCCTCCACCTCCGCCTCTGCCGC-------- 2251
C      GCGCCTGCCTGCTGCCGCGGGACNNCTGCCCGTCCACCTCGGCCGCCGCCGC-------- 1448
M      GCTCCTGCCTACTACCCCGGGACAGCCTGCCGGCCGCCCCGGCCACCGCCGC-------- 2134
       **  ****** ** ** ******  *   **  ** ** * *** * *****

P      CCGCCGCCGCCGCCGGGGCGGCTCCTGCGCTCTACCAGCCGCTTGGCCTTAACGGGCTCC 2592
H      -------CGCCGCCGGGGCGGCCCCCGCGCTCTACCCTGCACTCGGCCTCAACGGGCTCC 2304
C      --------------------------CGCCC--------------------------- 1453
M      ------------------AGCACCCGCCATCTACCAGCCGCTCGGCCTCAATGGGCTCC 2175
                                 *   *

P      CGCAGCTCGGCTACCAGGCCGCGGTACTCAAGGAGGGCCTGCCGCAGGTGTACCAGCCCT 2652
H      CGCAGCTCGGCTACCAGGCCGCCGTGCTCAAGGAGGGCCTGCCGCAGGTCTACCCGCCCT 2364
C      -----------------GCCGCCG----------GGGCCTGCCGCAGGTCTACCAGCCCT 1486
M      CGCAGCTGGGCTACCAGGCCGCGGTGCTCAAGGACAGCCTGCCCCAGGTCTACCCGCCAT 2235
                      ***** *           ******* ***** **** *** *
```

```
P     ATCTCAACTACCTGAGGCCGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTCGAGT 2712
H     ATCTCAACTACCTGAGGCCGGATTCAGAAGCCAGCCAGAGCCCACAATACAGCTTCGAGT 2424
C     ATCTCAACTACCTTAGGCCGGATTCAGAAGCCAGCCAGAGCCCACAGTACAGCTTTGAGT 1546
M     ACCTTAACTACCTGAGGCCAGATTCAGAAGCCAGCCAGAGCCCACAGTATGGCTTTGATT 2295
      * ** ******** ***** ************************** **  **** ** *

P     CATTACCTCAGAAGATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTG 2772
H     CATTACCTCAGAAGATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTG 2484
C     CATTACCTCAGAAGATTTGTTTAATCTGTGGGGATGAAGCATCAGGCTGTCATTATGGTG 1606
M     CCTTACCTCAGAAGATCTGCTTAATCTGCGGGGATGAAGCATCTGGCTGTCACTATGGCG 2355
      * ************** ** ******** ************** ******** ***** *

P     TCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAAAAGGGCAATGGAAGGGCAGCACAACT 2832
H     TCCTTACCTGTGGGAGCTGTAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCACAACT 2544
C     TCCTCACCTGTGGAAGCTGTAAGGTCTTCTTTAAAAGGGCAATGGAAGGACAGCATAACT 1666
M     TGCTTACCTGTGGGAGCTGCAAGGTCTTCTTTAAGAGGGCAATGGAAGGGCAGCATAACT 2415
      * ** ******** ***** ************** ************** ***** ****

P     ACTTATGTGCTGGAAGAAACGACTGCATTGTTGATAAAATCCGCAGAAAAAACTGCCCAG 2892
H     ACTTATGTGCTGGAAGAAATGACTGCATCGTTGATAAAATCCGCAGAAAAAACTGCCCAG 2604
C     ACTTATGTGCTGGAAGAAACGATTGCATTGTTGATAAAATCCGCAGAAAAAACTGCCCAG 1726
M     ATTTATGTGCTGGAAGAAATGACTGCATTGTTGATAAAATTCGCAGAAAAAACTGCCCAG 2475
      * ***************** ** ***** *********** ******************

P     CTTGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTCCTTGGAGGTCGAAAGTTTAAAA 2952
H     CATGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTCCTTGGAGGTCGAAAATTTAAAA 2664
C     CATGTCGCCTTAGAAAGTGCTGTCAGGCTGGCATGGTTCTTGGAGGCCGAAAGTTTAAAA 1786
M     CATGTCGTCTGAGAAAGTGTTGTCAGGCTGGCATGGTCCTTGGAGGTCGTAAGTTTAAGA 2535
      * ***** ** ******** ***************** ******** ** ** ***** *

P     AGTTCAATAAAGTTAGAGTTATGAGAGCACTAGATGCCGTTGCTCTCCCACAGTCGGGGG 3012
H     AGTTCAATAAAGTCAGAGTTGTGAGAGCACTGGATGCTGTTGCTCTCCCACAGCCAGTGG 2724
C     AGTTCAATAAAGTTAGAGTTATGAGAACACTAGATGCTGTTGCCCTCCCACAGCCGGTGG 1846
M     AGTTTAATAAAGTCCGAGTTATGAGAACCCTTGACGGTGTTGCTCTCCCCCAGTCGGTGG 2595
      **** ********  ***** ***** * ** ** *  ***** ***** *** * * **

P     GCATTCCAAATGAAAGTCAGGCCCTAAGCCAGAGAATCACTTTTTCACCAAATCAAGACT 3072
H     GCGTTCCAAATGAAAGCCAAGCCCTAAGCCAGAGATTCACTTTTTCACCAGGTCAAGACA 2784
C     GTATTCCAAACGAAAGCCAAGCCCTAAGCCAGAGAATCACTTTTTCACCAAGTCAAGACT 1906
M     GCCTTCCTAACGAGAGCCAGGCCCTGGGCCAGAGAATCACCTTTTCACCAAATCAAGAAA 2655
      *  **** ** ** ** ** *****  ******** **** *********  ******

P     TACAGCTGATCCCCCCGTTGATCAACTTGCTCATGAGCATTGAACCAGATGTGGTCTATG 3132
H     TACAGTTGATTCCACCACTGATCAACCTGTTAATGAGCATTGAACCAGATGTGATCTATG 2844
C     TGCAGTTGATCCCCCCACTGATCAACTTGCTCATGAGCATTGAACCAGATGTGGTCTATG 1966
M     TTCAACTGGTCCCGCCACTCATCAACCTGCTCATGAGCATTGAGCCTGATGTGGTCTATG 2715
      * **  ** * ** **  * ****** ** * *********** ** ****** ******

P     CAGGACATGACAACACCAAACCCGACACTTCGAGTTCTTTGCTGACAAGTCTGAATCAGC 3192
H     CAGGACATGACAACACAAAACCTGACACCTCCAGTTCTTTGCTGACAAGTCTTAATCAAC 2904
C     CAGGACATGACAACAGCAAACCTGATACCTCCAGCTCTCTGCTGACAAGTCTGAATCAGC 2026
M     CAGGGCATGACAACACAAAGCCTGACACTTCCAGCTCTTTGCTGACCAGTCTCAACCAAC 2775
      **** **********  ** ** ** ** ** ** *** ******* ***** ** ** *
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P     TGGGTGAAAGACAACTTCTTTCAGTAGTCAAGTGGTCTAAGTCACTGCCAGGTTTTCGAA 3252
H     TAGGCGAGAGGCAACTTCTTTCAGTAGTCAAGTGGTCTAAATCATTGCCAGGTTTTCGAA 2964
C     TTGGTGAGAGACAACTTCTTTCAGTGGTCAAGTGGTCTAAATCACTGCCAGGTTTTCGGA 2086
M     TAGGCGAGAGACAACTGCTTTCAGTAGTCAAATGGTCTAAATCTCTGCCAGGTTTCCGGA 2835
      * ** ** ** ***** ******** ***** ******** **  ********** ** *

P     ACTTACATATTGATGACCAAATAACTCTCATCCAGTATTCCTGGATGAGCTTAATGGTAT 3312
H     ACTTACATATTGATGACCAGATAACTCTCATTCAGTATTCTTGGATGAGCTTAATGGTGT 3024
C     ACTTACATATTGATGACCAAATAACTCTCATCCAGTATTCTTGGATGAGTTTAATGGTAT 2146
M     ACTTACACATTGATGACCAGATAACCCTGATTCAGTACTCCTGGATGAGCCTGATGGTGT 2895
      ******* *********** ***** ** ** ***** ** ********  * ***** *

P     TTGGACTAGGATGGAGATCCTATAAACATGTCAGTGGACAGATGCTTTATTTTGCACCTG 3372
H     TTGGTCTAGGATGGAGATCCTACAAACACGTCAGTGGGCAGATGCTGTATTTTGCACCTG 3084
C     TTGGACTAGGATGGAGATCTTATAAGCATGTCAGTGGACAGATGCTATATTTTGCGCCTG 2206
M     TTGGCCTGGGGTGGAGGTCGTACAAGCATGTCAGTGGACAGATGCTATATTTTGCACCTG 2955
      **** ** ** ***** ** ** ** ** ******** ******** ******** ****

P     ATCTAATACTAAATGAACAGCGGATGAAGGAATCA-TCAT----------TCTATTCATT 3421
H     ATCTAATACTAAATGAACAGCGGATGAAAGAATCA-TCAT----------TCTATTCATT 3133
C     ATCTAATACTAAATGAAAGCTGGGGTGTGCAACCAGCCACGACCCGCGTGTCTCTCTGCC 2266
M     ATCTAATCCTAAATGAGCAGAGGATGAAGGAGCTG-TCAT----------TCTACTCGCT 3004
      ******* ********     **       *      **           ***

P     A-TGCCTTACCATGTGGCAGATCCCACAGGAGTTTGTGAA--GCTTCAAGTTAGCCAAGA 3478
H     A-TGCCTTACCATGTGGCAGATCCCACAGGAGTTTGTCAA--GCTTCAAGTTAGCCAAGA 3190
C     AGTGTCCTCGCAGCTGCTCTCTCCTGCACCACATTTTTAACTGCTACAACTTTGCTCTC- 2325
M     G-TGCCTTACCATGTGGCAAATCCCACAGGAGTTTGTCAA--ACTCCAGGTGACCCATGA 3061
        ** * *  **  **     ***  **  *  ** * **   ** **  *   *

P     AGAGTTTCTCTGTATGAAAGTATTGCTACTTCTTAATACAATTCCTTTGGAAGGACTAAG 3538
H     AGAGTTCCTCTGTATGAAAGTATTGTTACTTCTTAATACAATTCCTTTGGAAGGGCTACG 3250
C     ----CTTTCTTGGATTAAGTTTTTAAAATGTGATAGTAAAGTTCCTTTAGAAGGACTAAG 2381
M     GGAATTCCTCTGTATGAAAGTCTTACTACTTCTTAACACAATTCCTTTGGAAGGACTGAG 3121
           *    ** ** **  * **   *  *  **  * * ******* ***** **  *

P     AAGTCAAAACCAGTTTGAAGAGATGAGATCAAGCTACATTAGAGAGCTCATCAAGGCAAT 3598
H     AAGTCAAACCCAGTTTGAGGAGATGAGGTCAAGCTACATTAGAGAGCTCATCAAGGCAAT 3310
C     AAGTCAAAACCAGTTTGAAGAGATGAGATCAAGTTACATTAGAGAGCTCATCAAGGCAAT 2441
M     GAGTCAAAGCCAGTTTGAAGAGATGAGATCAAGCTATATCCGCGAATTGATCAAGGCAAT 3181
       ******* ********* ******** ***** ** **  * **  * ***********

P     TGGTTTGAGACAAAAAGGAGTCGTCCCTAGCTCACAGCGTTTCTACCAGCTTACAAAACT 3658
H     TGGTTTGAGGCAAAAAGGAGTTGTGTCGAGCTCACAGCGTTTCTATCAACTTACAAAACT 3370
C     TGGTTTGAGGCAAAAAGGAGTTGTCCCTAGCTCACAGCGTTTCTATCAGCTTACAAAACT 2501
M     TGGTTTAAGACAGAAAGGGGTTGTCCCCAGCTCACAGCGCTTCTACCAACTCACAAAACT 3241
      ****** ** ** ***** ** **  * *********** ***** ** ** ********

P     TCTTGATAACTTGCATGAT-----------CTTGTCAAACAACTTCATCTGTACTGCTT 3706
H     TCTTGATAACTTGCATGAT-----------CTTGTCAAACAACTTCATCTGTACTGCTT 3418
C     TCTTGATAACTTGCATGATATAAGTATTTTGCTTGTCAAACAACTTCATCTGTATTGCTT 2561
M     TCTCGACAGCTTGCATGAT-----------CTTGTGAAACAGCTCCACCTGTACTGCTT 3289
      *** ** * **********            ***** ***** ** ** ***** *****
```

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P      GAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAATTTCCAGAAATGATGTCTGAGGT 3766
H      GAATACATTTATCCAGTCCCGGGCACTGAGTGTTGAATTTCCAGAAATGATGTCTGAAGT 3478
C      GAATACATTTATCCAGTCCTGGGCACTGAGTGTTGAATTTCCAGAAATGATGTCTGAGGT 2621
M      GAATACATTCATCCAATCCCGGACACTGGCTGTGGAATTTCCGGAAATGATGTCTGAAGT 3349
       ********* ***** *** ** *****  *** ******** ************** **

P      TATTGCTGCACAATTACCCAAGATCTTGGCAGGGATGGTGAAACCCCTTCTCTTTCATAA 3826
H      TATTGCTGCACAATTACCCAAGATATTGGCAGGGATGGTGAAACCCCTTCTCTTTCATAA 3538
C      TATTGCTGCACAATTACCCAAGATCTTGGCAGGGATGGTGAAATCTCTTCTCTTTCATAA 2681
M      TATTGCTGCCCAGTTGCCCAAGATCCTGGCGGGCATGGTGAAGCCGCTCCTCTTTCACAA 3409
       ********* ** ** ********  **** ** ********  * ** ******** **

P      AAAGTGAA----TGCCATTTTTATCTTT-AAAAAATTAAACTTTGTGGTATATCTGTTTT 3881
H      AAAGTGAA----TGTCATCTTTTTCTTTTAAAGAATTAAATTTTGTGGTATGTCT--TTT 3592
C      AAAGTGA---------------------------------------------------- 2688
M      AAAGTGATGACTTTTCTTTGTTTTCTTTGTGAAAATTGAATCTTGTGGTGTGCCA-TCTT 3468
       *******

P      CGTTTTTGTCAGGATTATGAGGTCTTGAGTTTTTATAATGTTCTC---AAAGCCCTACAT 3938
H      TGTTTTGGTCAGGATTATGAGGTCTTGAGTTTTTATAATGTTCTTCTGAAAGCCTTACAT 3652
C      ------------------------------------------------------------
M      TTTCTCAGTCAGGATGGTCAGGTCATGTGTCTTTATAATGCCTTTCTGATAGCCTTACAT 3528

P      TTATAACATATTGTACTGTGTAAATTTCAGAGGAAAAATGTGAGGGTGAATTGGATTTGC 3998
H      TTATAACAT--CATAGTGTGTAAATTTAAAAGAAAAATTGTGAGGTTCTAATTATTTTCT 3710
C      ------------------------------------------------------------
M      GTATGCACTATCACACTG--TAAAGTTAGGAGAAAACCAATGACATTC---TGACTTTCT 3583

P      TTTATAAAGCATTATTTGAATTTTT----AGTGTTTTTGCGTACCCATATTTTCTTTAAG 4054
H      TTTATAAAGTATAATTAGAATGTTT----AACTGTTTTGTTTACCCATATTTTCTTGAAG 3766
C      ------------------------------------------------------------
M      TTTGTAATGTCTAAGCAGAGTTTTTTTTTAAGTGTTGTGTGCACCCATATTTT----AAG 3639

P      AGTTTACACGATT---GAAAAAGTACTAAGTT-GATTGTTAAAGTTAA---TCCTATCCG 4107
H      AATTTACAAGATT---GAAAAAGTACTAAA----ATTGTTAAAGTAAACTATCTTATCCA 3819
C      ------------------------------------------------------------
M      AGTTTACATGGTTTTTAAAAAATGACTAAAATTGATTGTACGAGTAAACTATATTTCACC 3699

P      TATCATTTCATACCATTTAGGTGAG-ATTTTTAACTTTCACAGCTAACAGATCCTCTACT 4166
H      TATTATTTCATACCATGTAGGTGAGGATTTTTAACTTTTGCATCTAACAAATCATCGACT 3879
C      ------------------------------------------------------------
M      CATTATTTCAAACTTTTCAGATGAGTGTAATCAACTTTTTCATCTGACAGATCTCCATGA 3759

P      TTAGAGGAAAAAAAATCT-----TACACATAAAAATAAAAAGCTATTATATATGTTCCTTC 4221
H      TAAGAG--AAAAAATCT-----TACATGTAATAACACAAAGCTATTATAT--GTTATTTC 3930
C      ------------------------------------------------------------
M      GAAGGGAAGAAACACCCCTCCCTACCTTTAATTACAACAGGCTACTACACAGGCTATGGC 3819
```

```
P      TATGTAGCTCCCTTTGTCTCCCTGATTATATTTACAAAACTGAAACTTTAAAATGGTATG 4281
H      TAGGTAACTCCCTTTGTGTC---AATTATATTTCCAAAAATGAACCTTTAAAATGGTATG 3987
C      ------------------------------------------------------------
M      TATGTAGTTCCTTTGGTCTCCATGCTTATATTTCCAAAAATGA-CTCTTAAAATGGCAGT 3878

P      CAAAACTTGTCTATATGAGTGTACATACATTTTTTTGTGAGAAGGATATTTATAATCTTA 4341
H      CAAAATTT-----------TGTCTATATATATTTGTGTGAGGAGGAAATTCATAACTTTC 4036
C      ------------------------------------------------------------
M      CAAAATGTG---------ATGTGCA-ATAGATTT---TCAGAGGGCTTTTAGTGAA---- 3921

P      ATCAGTTTTTCCAAAGGGTTTTTAATGCAAAAATATATATATACAGAAAAAGAGTTAACA 4401
H      CTCAGATTTTCAAAAGTATTTTTAATGCAAAAA---------ATGTAGAAAGAGTTTAAA 4087
C      ------------------------------------------------------------
M      ---AAA----CAAAA-------CAAAACAAACA-----------ACAACAACAACAAAAC 3956

P      ATTCTTTAATTAGAT-GATACTCCTCAAACTAGGAAAACCAGCTTATATGTTAAGACTGT 4460
H      ACCACTAAAATAGATTGATGTTCTTCAAACTAGGCAAAACAACTCATATGTTAAGACCAT 4147
C      ------------------------------------------------------------
M      AGAGATGAAATGGGTTGATGTTTTTAACTCTAGGCAAAC-------TGTGTTCAGGGTGC 4009

P      TTCCCAGATTGGAAACACAAATCTCTAGGGAAGTTATTAAGTAGAGCCATAGCAG----- 4515
H      TTTCCAGATTGGAAACACAAATCTCTTAGGAAGTTAATAAGTAGATTCATATCATTATGC 4207
C      ------------------------------------------------------------
M      CTGCCTGAT------AACAGATTGATGACAGAGTTAATG-GCAG--CTATACAGTTGCTG 4060
```

Figure 3.9 Porcine progesterone receptor (PGR) mRNA and 934 amino acid encoded protein sequence. The mRNA nucleotide sequence (5' – 3', top line) and corresponding amino acid sequence (N terminus – C terminus, bottom line). Methionine (M) start sites for PGR-B, PGR-A and PGR-C isoforms are indicated in bold and underlined text. The open reading frame was identified and codons translated to a putative amino acid sequence by the ORF Finder program (NCBI).

atgactgagctgaaggcaaaggatccccgggctccccacgtggcgggcagagcgccctcccccacccagctcgggacgctgggacgcccagacacaggccccttttcaggcgagccagacc
M T E L K A K D P R A P H V A G R A P S P T Q L G T L G R P D T G P F Q A S Q T
tcggaagcgtcgcccgcagcctcggccatacccctctccctggacgggctactcttccctgggccctgccagggacaggaaccagacgggaagacgcaggaccagcagtcgctgtcagac
S E A S P A A S A I P L S L D G L L F P G P C Q G Q E P D G K T Q D Q Q S L S D
gtggaggggcgtatcccagagttgaagctacagagggtgctggaggtggcagctctagaccctcggaaaaagacaccgggctgctggacagtgtcttggacacgctactagcgccctca
V E G A Y P R V E A T E G A G G G S S R P S E K D T G L L D S V L D T L L A P S
ggtcccgggcagagccacgccagccctcccgcctgcgaagccaccagcccttggtgcttgtttggctctgagcttccccaggacgctcgggttgccccttccacccagggagtattgccc
G P G Q S H A S P P A C E A T S P W C L F G S E L P Q D A R V A P S T Q G V L P
ctgctcatgagccggccagagggcaaggctggcgacagctccgggacggcagccgcccataaagtgctatccaggggtctggcaccgtcccggcagctgctgccctcgactgccgggagc
L L **M** S R P E G K A G D S S G T A A A H K V L S R G L A P S R Q L L P S T A G S
catcactggccggcggccgcagtgaagccctctccgcagcccgccgtggtggaggtggaggaggaggatgactccgagtccgagggctccatggttccacttctgaagggtaaaccccgg
H H W P A A A V K P S P Q P A V V E V E E E D D S E S E G S M V P L L K G K P R
cctgcaggaggcacggctgccggaacaggaaccccgaccggtgctcctgggacggccgcaggaggcgtcgccctggtccccaaggaagatgcccgcttctcggcgcccagggggcgccctg
P A G G T A A G T G T P T G A P G T A A G G V A L V P K E D A R F S A P R G A L
gcggagcacgatgcgtcaggggcgcccgggcgctcccctctggccactacggtgatggattttatccacgtgcccatcctgccgctcaacacggcctttctggccgcccgcacccggcaa
A E H D A S G A P G R S P L A T T V M D F I H V P I L P L N T A F L A A R T R Q
ctgctagaggggataactatgacggcggggccccggctgtcagcgcctttgcaccgccgcggggctcgccctcggccccgtccgcctccgtcaccgccggcgacttccccgactgcgcg
L L E G D N Y D G G A P A V S A F A P P R G S P S A P S A S V T A G D F P D C A
tactctgcagacgacgagcccaaggacgacgcattcccgctctatggcgacttccagccgcccgccctgaagatcaaggaagaggaggaaggcgccgaggccgccgcgcgctctccgcgg
Y S A D D E P K D D A F P L Y G D F Q P P A L K I K E E E E G A E A A A R S P R
tcgtacctggtggcgggtgccaaccctgccgtcttcccggattttccgctggcgccgccgtcgctgcctccgcgagcgtcgtccagacctggggaagcctcggcggcggtcgcacccacc
S Y L V A G A N P A V F P D F P L A P P S L P P R A S S R P G E A S A A V A P T
agtgcctcggtatcgtcggcgtcctcgtcggggtcggccttggagtgcatgttgtataaggcggagggcgcgccgccccaacagggcccgttcccgcctccgccctgcaagccgccgagc
S A S V S S A S S S G S A L E C M L Y K A E G A P P Q Q G P F P P P P P C K P P S
gccggcgcctgcctgctgccgcgggacagcctgccgtccacctcggcctccgccgccgccgccgccgccgccgccgccgcgggcggctcctgcgctctaccagccgcttggcctcaacggg
A G A C L L P R D S L P S T S A S A A A A A A A A A A G A A P A L Y Q P L G L N G
ctcccgcagctcggctaccaggccgcggtactcaaggagggcctgccgcaggtgtaccagccctatctcaactacctgaggccggattcagaagccagccagagcccacagtacagcttc
L P Q L G Y Q A A V L K E G L P Q V Y Q P Y L N Y L R P D S E A S Q S P Q Y S F
gagtcattacctcagaagatttgtttaatctgtggggatgaagcatcaggctgtcattatggtgtccttacctgtgggagctgtaaggtctttcttaaaagggcaatggaagggcagcac
E S L P Q K I C L I C G D E A S G C H Y G V L T C G S C K V F L K R A **M** E G Q H
aactacttatgtgctggaagaaacgactgcattgttgataaaatccgcagaaaaaactgcccagcttgtcgccttagaaagtgctgtcaggctggcatggtccttggaggtcgaaagttt
N Y L C A G R N D C I V D K I R R K N C P A C R L R K C C Q A G M V L G G R K F
aaaaagttcaataaagttagagttatgagagcactagatgccgttgctctcccacagtcgggggggcattccaaatgaaagtcaggccctaagccagagaatcacttttcaccaaatcaa
K K F N K V R V M R A L D A V A L P Q S G G I P N E S Q A L S Q R I T F S P N Q
gacttacagctgatccccccgttgatcaacttgctcatgagcattgaaccagatgtggtctatgcaggacatgacaacaccaaacccgacacttcgagttctttgctgacaagtctgaat
D L Q L I P P L I N L L M S I E P D V V Y A G H D N T K P D T S S S L L T S L N
cagctgggtgaaagacaacttctttcagtagtcaagtggtctaagtcactgccaggttttcgaaacttacatattgatgaccaaataactctcatccagtattcctggatgagcttaatg
Q L G E R Q L L S V V K W S K S L P G F R N L H I D D Q I T L I Q Y S W M S L M
gtatttggactaggatggagatcctataaacatgtcagtggacagatgctttattttgcacctgatctaatactaaatgaacagcggatgaaggaatcatcattctattcattatgcctt
V F G L G W R S Y K H V S G Q M L Y F A P D L I L N E Q R M K E S S F Y S L C L
accatgtggcagatcccacaggagtttgtgaagcttcaagttagccaagaagagtttctctgtatgaaagtattgctacttcttaatacaattcctttggaaggactaagaagtcaaaac
T M W Q I P Q E F V K L Q V S Q E E F L C M K V L L L L N T I P L E G L R S Q N
cagtttgaagagatgagatcaagctacattagagagctcatcaaggcaattggtttgagacaaaaaggagtcgtccctagctcacagcgtttctaccagcttacaaaacttcttgataac
Q F E E M R S S Y I R E L I K A I G L R Q K G V V P S S Q R F Y Q L T K L L D N
ttgcatgatcttgtcaaacaacttcatctgtactgcttgaatacatttatccagtcccgggcactgagtgttgaatttccagaaatgatgtctgaggttattgctgcacaattacccaag
L H D L V K Q L H L Y C L N T F I Q S R A L S V E F P E M M S E V I A A Q L P K
atcttggcagggatggtgaaacctcttctctttcataaaaagtgaatgccatttttatct
I L A G M V K P L L F H K K - M P F L S

Figure 3.10 Porcine progesterone receptor (PGR) predicted 934 amino acid protein sequence (NP001159960.1) inferred from mRNA sequence (GQ903679.1). Amino acid sequence generated by NCBI ORF Finder program listed from N terminus – C terminus. Methionine (M) start sites for PGR-B, PGR-A and PGR-C isoforms are indicated in bold and underlined text. Protein domains are shown as determined in human PGR protein: A/B variable region (unformatted), C DNA binding domain (italics, underlined), D hinge domain (wavy underline), and E hormone binding domain (double underline).

```
  1 Mtelkakdpr aphvagraps ptqlgtlgrp dtgpfqasqt seaspaasai plsldgllfp
 61 gpcqgqepdg ktqdqqslsd vegayptvea tegagggssr psekdtglld svldtllaps
121 gpgqshaspp aceatspwcl fgselpqdar vapstqgvlp llMsrpegka gdssgtaaah
181 kvlsrglaps rqllpstags hhwpaaavkp spqpavveve eeddsesegs mvpllkgkpr
241 paggtaagtg tptgapgtaa ggvalvpked arfsaprgal aehdasgapg rsplattvmd
301 fihvpilpln taflaartrq llegdnydgg apavsafapp rgspsapsas vtagdfpdca
361 ysaddepkdd afplygdfqp palkikeeee gaeaaarspr sylvaganpa vfpdfplapp
421 slpprassrp geasaavapt sasvssasss gsalecmlyk aegappqqgp fppppckpps
481 agacllprds lpstsasaaa aaaaaagaap alyqplglng lpqlgyqaav lkeglpqvyq
541 pylnylrpds easqspqysf eslpqkicli cgdeasgchy gvltcgsckv ffkraMegqh
601 nylcagrndc ivdkirrknc pacrlrkccq agmvlggrkf kkfnkvrvmr aldavalpqs
661 ggipnesqal sqritfspnq dlqlipplin llmsiepdvv yaghdntkpd tsslltsln
721 qlgerqllsv vkwskslpgf rnlhiddqit liqyswmslm vfglgwrsyk hvsgqmlyfa
781 pdlilneqrm kessfyslcl tmwqipqefv klqvsqeefl cmkvllllnt ipleglrsqn
841 qfeemrssyi relikaiglr qkgvvpssqr fyqltkslldn lhdlvkqlhl yclntfiqsr
901 alsvefpemm seviaaqlpk ilagmvkpll fhkk
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Figure 3.11 Alignment of porcine PGR protein amino acid sequences inferred from mRNA (GQ903679.1) and genomic (CU640401.2) sequences. Alignment generated by BLASTP program (NCBI). Query denotes protein derived from mRNA sequence; Sbjct denotes protein derived from genomic sequence and location (base) in the sequence are marked. The middle line contains the consensus protein sequence. There are no gaps in alignment and 918 of 934 amino acid residues (98%) are synonymous. Spaces indicate non-conservative amino acid substitutions; plusses (+) indicate conservative amino acid substitutions. Protein domains are shown as determined in human PGR protein: A/B variable region (unformatted), C DNA binding domain (italics, underlined), D hinge domain (wavy underline), and E hormone binding domain (double underline).

```
Query  1    MTELKAKDPRAPHVAGRAPSPTQLGTLGRPDTGPFQASQTSEASPAASAIPLSLDGLLFP  60
            MTELKAKDPRAPHVAGRAPSPTQLGTLGRPDTGPFQASQTSEASPAASAIPLSLDGLLFP
Sbjct  1    MTELKAKDPRAPHVAGRAPSPTQLGTLGRPDTGPFQASQTSEASPAASAIPLSLDGLLFP  60

Query  61   GPCQGQEPDGKTQDQQSLSDVEGAYPTVEATEGAGGGSSRPSEKDTGLLDSVLDTLLAPS  120
            GPCQGQEPDGKTQDQQSLSDVEGAYP VEATEGAGGGSSRPSEKDTGLLDSVLDTLLAPS
Sbjct  61   GPCQGQEPDGKTQDQQSLSDVEGAYPRVEATEGAGGGSSRPSEKDTGLLDSVLDTLLAPS  120

Query  121  GPGQSHASPPACEATSPWCLFGSELPQDARVAPSTQGVLPLLMSRPEGKAGDSSGTAAAH  180
            GPGQSHASPPACEATSPWCLFGSELPQDARVAPSTQGVLPLLMSRPEGKAGDSSGTAAAH
Sbjct  121  GPGQSHASPPACEATSPWCLFGSELPQDARVAPSTQGVLPLLMSRPEGKAGDSSGTAAAH  180

Query  181  KVLSRGLAPSRQLLPSTAGSHHWPAAAVKPSPQPAVVEVEEEDDSESEGSMVPLLKGKPR  240
            KVLSRGLAPSRQLLPSTAGSHHWPAAAVKPSPQPAVVEVEEEDDSESEGSMVPLLKGKPR
Sbjct  181  KVLSRGLAPSRQLLPSTAGSHHWPAAAVKPSPQPAVVEVEEEDDSESEGSMVPLLKGKPR  240

Query  241  PAGGTAAGTGTPTGAPGTAAGGVALVPKEDARFSAPRGALAEHDASGAPGRSPLATTVMD  300
            PAGGTAAGTGTPTGAPGTAAGGVALVPKEDARFSAPRGALAEHDASGAPGRSPLATTVMD
Sbjct  241  PAGGTAAGTGTPTGAPGTAAGGVALVPKEDARFSAPRGALAEHDASGAPGRSPLATTVMD  300

Query  301  FIHVPILPLNTAFLAARTRQLLEGDNYDGGAPAVSAFAPPRGSPSAPSASVTAGDFPDCA  360
            FIHVPILPLNTAFLAARTRQLLEGDNYDGGAPAVSAFAPPRGSPSAPSASVTAGDFPDCA
Sbjct  301  FIHVPILPLNTAFLAARTRQLLEGDNYDGGAPAVSAFAPPRGSPSAPSASVTAGDFPDCA  360

Query  361  YSADDEPKDDAFPLYGDFQPPALKIKEEEEGAEAAARSPRSYLVAGANPAVFPDFPLAPP  420
            YSADDEPKDDAFPLYGDFQPPALKIKEEEEGAEAAARSPRSYLVAGA PAVFPDFPLAPP
Sbjct  361  YSADDEPKDDAFPLYGDFQPPALKIKEEEEGAEAAARSPRSYLVAGATPAVFPDFPLAPP  420

Query  421  SLPPRASSRPGEASAAVAPTSASVSSASSSGSALECMLYKAEGAPPQQGPFPPPPCKPPS  480
            SLPPRASSRPGEASAAVAPTSASVSSASSSGSALECMLYKAEGAPPQQGPFPPPPCKPPS
Sbjct  421  SLPPRASSRPGEASAAVAPTSASVSSASSSGSALECMLYKAEGAPPQQGPFPPPPCKPPS  480

Query  481  AGACLLPRDSLPSTSASAAAAAAAAAGAAPALYQPLGLNGLPQLGYQAAVLKEGLPQVYQ  540
            AGACLLPRDSLPSTSASAAAAAAAAAGAAPALYQPLGLNGLPQLGYQAAVLKEGLPQVYQ
Sbjct  481  AGACLLPRDSLPSTSASAAAAAAAAAGAAPALYQPLGLNGLPQLGYQAAVLKEGLPQVYQ  540

Query  541  PYLNYLRPDSEASQSPQYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFFKRAMEGQH  600
            PYLNYLR DSEASQ P+QYSFESLPQ +C+ICGDEASGCHYGVLTCGSCKVFFKRAMEGQH
Sbjct  541  PYLNYLRTDSEASQRPQYSFESLPQNLCIICGDEASGCHYGVLTCGSCKVFFKRAMEGQH  600

Query  601  NYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKKFNKVRVMRALDAVALPQS  660
            NYLCAGRNDCI+DKIRRKNCPACRL+KC QAGM+LGGRKFKK  KVRVMRALDAVALP S
Sbjct  601  NYLCAGRNDCIIDKIRRKNCPACRLQKCLQAGMILGGRKFKKSIKVRVMRALDAVALPHS  660

Query  661  GGIPNESQALSQRITFSPNQDLQLIPPLINLLMSIEPDVVYAGHDNTKPDTSSSLLTSLN  720
            GGIPNESQALSQRITFS NQDLQLIPPLINLLMSIEPDVVYAGHDNTKPDTSSSLLTSLN
Sbjct  661  GGIPNESQALSQRITFSANQDLQLIPPLINLLMSIEPDVVYAGHDNTKPDTSSSLLTSLN  720

Query  721  QLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFA  780
            QLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFA
Sbjct  721  QLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQMLYFA  780

Query  781  PDLILNEQRMKESSFYSLCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQN  840
            PDLILNEQRMKESSFYSLCL MWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQN
Sbjct  781  PDLILNEQRMKESSFYSLCLNMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGLRSQN  840

Query  841  QFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSR  900
            QFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSR
Sbjct  841  QFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDLVKQLHLYCLNTFIQSR  900

Query  901  ALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK  934
            ALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK
Sbjct  901  ALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK  934
```

to human PGR protein within the C, D, and E regions (Figure 3.12). The full-length porcine PGR protein was 80% identical to the mouse PGR sequence (NP032855.2) and 72% identical to the predicted bovine PGR sequence (XP583951.5; Table 3.3). Identity is nearly perfect between all four species beginning around residue 500, indicating that the C terminus of the variable domain, the DBD, hinge and LBD are highly conserved among higher order mammals. Moderate amino acid identity was observed in the variable domain, but appears to be decreased between residues 400 and 500 (Figure 3.13).

The generated mRNA sequence from multiple animals, once validated as described above, and genomic sequence from GenBank were compared to identify polymorphic sites and sequence gaps within the domesticated pig population. Eleven polymorphic sites and 3 gaps were identified between GQ903679.1 mRNA and CU640401.2 genomic PGR nucleotide sequences (Table 3.4). Four single nucleotide polymorphisms (SNPs) were discovered within exon 1. Two SNPs were located in the 5' UTR and thus were non-coding. The other 2 SNPs were located in the protein coding sequence: 1288 (G/C) results in an arginine to threonine substitution, 2582 (C/T) does not alter amino acid identity (leucine). The polymorphism 2401 (T/-) shifts the reading frame. The two other gaps between the sequences occur in exon 2. Both 2801 (-/T) and 2805 (T/-) shift the open reading frame of the transcript, suggesting sequencing errors in either the genomic or mRNA sequence. Two SNPs in exon 7 result in amino acid substitutions. The 3513 (A/C) SNP encodes an asparagine to histidine substitution. The 3519 (A/G) SNP encodes an isoleucine to valine substitution. The final 6 polymorphisms are located in exon 8. Four of them are non-coding: 3895 (A/G), 4294 (C/A), 4354 (A/G)

and 4470 (T/G) and one is a gap 3971 (-/A), probably representing a sequencing error in the genomic sequence. Two of these SNPs were represented within our sample population: 2582 (C/T; Figure 3.14), and 4294 (A/C; Figure 3.15).

Figure 3.12 Amino acid sequence alignment of predicted porcine and human (NP000917.3) PGR from the first methionine residue. Alignment generated by ClustalW2 software. Protein domains are shown: A/B variable region (unformatted), C DNA binding domain (italics, underlined), D hinge domain (wavy underline), and E hormone binding domain (double underline). Porcine mRNA sequence was generated from cDNA as described above and sequence was translated into the predicted porcine PGR protein sequence. Stars (*) indicate synonymous residues between the sequences, colons (:) indicate highly conservative residues, periods (.) indicate weakly conservative residues, spaces indicate non-conservative residues and dashes indicate gaps in the sequence. Overall homology between the sequences is 84%, with 75% identity in the A/B variable region alone and 97% identity in the C, D, and E regions combined.

```
PIG     MTELKAKDPRAPHVAGRAPSP-TQLGTLGRPDTGPFQASQTSEASPAASAIPLSLDGLLF 59
HUMAN   MTELKAKGPRAPHVAGGPPSPEVGSPLLCRPAAGPFPGSQTSDTLPEVSAIPISLDGLLF 60
        *******.******** .*** .    * ** :*** .****:: * .****:*******

PIG     PGPCQGQEP-DGKTZDQQSLSDVEGAYPRVEATEGAGGGSSRPSEKDTGLLDSVLDTLLA 118
HUMAN   PRPCQGQDPSDEKTQDQQSLSDVEGAYSRAEATRGAGGSSSSPPEKDSGLLDSVLDTLLA 120
        * *****:* * ** ***********.*.***.****.** *.***:************

PIG     PSGPGQSHASPPACEATSPWCLFGSELPQDARVAPSTQGVLPLLMSRPEGKAGDSSGTAA 178
HUMAN   PSGPGQSQPSPPACEVTSSWCLFGPELPEDPPAAPATQRVLSPLMSRSGCKVGDSSGTAA 180
        *******:.******.**.*****.***:*. .**:** **. ****.  *.********

PIG     AHKVLSRGLAPSRQLLPSTAGSHHWPAAAVKPSPQPAVVEVEEEDDSESEGSMVPLLKGK 238
HUMAN   AHKVLPRGLSPARQLLLPASESPHWSGAPVKPSPQAAAVEVEEEDGSESEESAGPLLKGK 240
        *****.***:*:**** .:: * **..*.******.*.*******.**** *  ******

PIG     PRPAGGTAAGTGTPTGAPGTAAGGVALVPKEDARFSAPRGALAEHDASGAPGRSPLATTV 298
HUMAN   PRALGGAAAGGGAAAVPPGAAAGGVALVPKEDSRFSAPRVALVEQDAPMAPGRSPLATTV 300
        **. **:*** *:.: .**:************:****** **.*:**. ***********

PIG     MDFIHVPILPLNTAFLAARTRQLLEGDNYDGGAPAVSAFAPPRGSPSAPSASVTAGDFPD 358
HUMAN   MDFIHVPILPLNHALLAARTRQLLEDESYDGGAGAASAFAPPRSSPCASSTPVAVGDFPD 360
        ************ *:**********.::***** *.*******.**.*.*:.*:.*****

PIG     CAYSADDEPKDDAFPLYGDFQPPALKIKEEEEGAEAAARSPRSYLVAGANPAVFPDFPLA 418
HUMAN   CAYPPDAEPKDDAYPLYSDFQPPALKIKEEEEGAEASARSPRSYLVAGANPAAFPDFPLG 420
        ***..* ******:***.******************:***************.******.

PIG     PP-SLPPRAS-SRPGEASAAVAPTSASVSSASSSGSALECMLYKAEGAPPQQGPFPPPPC 476
HUMAN   PPPPLPPRATPSRPGEAAVTAAPASASVSSASSSGSTLECILYKAEGAPPQQGPFAPPPC 480
        ** .*****: ******:.:.**:************:***:**************.****

PIG     KPPSAGACLLPRDSLPSTSASAAAAAAAAAGAAPALYQPLGLNGLPQLGYQAAVLKEGLP 536
HUMAN   KAPGASGCLLPRDGLPSTSAS-----AAAAGAAPALYPALGLNGLPQLGYQAAVLKEGLP 535
        *.*.*..******.*******     *********** .********************

PIG     QVYQPYLNYLRPDSEASQSP*QYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFLKRAM* 596
HUMAN   QVYPPYLNYLRPDSEASQSP*QYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFFKRAM* 595
        *** **************************************************:****

PIG     *EGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKK*FNKVRVMRALDAVA 656
HUMAN   *EGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKK*FNKVRVVRALDAVA 655
        **************************************************:*******

PIG     LPQSGGIPNESQALSQRITFSPNQDLQLIPPLINLLMSIEPDVVYAGHDNTKPDTSSSLL 716
HUMAN   LPQPVGVPNESQALSQRFTFSPGQDIQLIPPLINLLMSIEPDVIYAGHDNTKPDTSSSLL 715
        ***. *:**********:****.**:*****************:****************

PIG     TSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQM 776
HUMAN   TSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSGQM 775
        ************************************************************

PIG     LYFAPDLILNEQRMKESSFYSLCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGL 836
HUMAN   LYFAPDLILNEQRMKESSFYSLCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLNTIPLEGL 835
        ************************************************************
```

```
PIG     RSQNQFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDLVKQLHLYCLNTF 896
HUMAN   RSQTQFEEMRSSYIRELIKAIGLRQKGVVSSSQRFYQLTKLLDNLHDLVKQLHLYCLNTF 895
        ***.************************.****************************

PIG     IQSRALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK 934
HUMAN   IQSRALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK 933
        *************************************
```

Table 3.3 Matrix of amino acid identity (%) between mouse (NP032855.2), predicted pig, human (NP000917.3), and predicted cow (XP583951.5) PGR protein from the first methionine residue (PGR-B). Alignment was generated and identity percentage calculated by ClustalW2 software.

	Human	**Mouse**	**Pig**	**Cow**
Human		85	85	80
Mouse	85		80	75
Pig	85	80		72
Cow	80	75	72	

Figure 3.13 Amino acid multiple sequence alignment of mouse (**M**; NP032855.2), predicted pig (**P**), human (**H**; NP000917.3), and predicted cow (**C**; XP583951.5) PGR protein from the first methionine residue (PGR-B). Protein domains are shown: A/B variable region (unformatted), C DNA binding domain (italics, underlined), D hinge domain (wavy underline), and E hormone binding domain (double underline). Stars (*) indicate synonymous residues between the sequences, colons (:) indicate highly conservative residues, periods (.) indicate weakly conservative residues, spaces indicate non-conservative residues and dashes indicate gaps in the sequence. Porcine mRNA sequence was generated from cDNA as described above and sequence was translated into the predicted porcine PGR protein sequence. Homology among the species is lowest in the variable domain and higher in the C, D, and E domains, with humans, mice and pigs nearly identical at the C terminus.

```
H     MTELKAKGPRAPHVAGGPPSP-EVGSPLLCRPAAGPFPGSQTSDTLPEVSAIPISLDGLL 59
M     MTELQAKDPQVLHTSGASPSPPHIGSPLLARLDSGPFQGSQHSDVSSVVSPIPISLDGLL 60
P     MTELKAKDPRAPHVAGRAPSPTQLGT--LGRPDTGPFQASQTSEASPAASAIPLSLDGLL 58
C     MTELKAKGPRAPHVAGTAPSPTQAGAPLRGRPDAGPFQAGEASGPTPTASGLPLSLDGLI 60
      ****:**.*:. *.:* .*** . *:    *  :*** ..: *   . .* :*:*****:

H     FPRPCQGQDPSDEKTQDQQSLSDVEGAYSRAEATRGAGGSSSSPPEKDSGLLDSVLDTLL 119
M     FPRSCRGPELPDGKTGDQQSLSDVEGAFSGVEATHREGGRNSRAPEKDSRLLDSVLDSLL 120
P     FPGPCQGQE-PDGKTQDQQSLSDVEGAYPTVEATEGAGGGSSRPSEKDTGLLDSVLDTLL 117
C     FPRSCQAQD-LDGKTPDPESLADVEGAYSGGEAAEG---AAARPPEKDGGLLDSVLDTLL 116
      ** .*:. :  * ** * :**:*****:.  **:.     : ..***  *******:**

H     APSGPGQSQPSPPACEVTSSWCLFGPELPEDPPAAPATQRVLSPLMSRSGCKVGDSSGTA 179
M     TPSGTEQSHASPPACEAITSWCLFGPELPEDPRSVPATKGLLSPLMSRPEIKAGDSSGTG 180
P     APSGPGQSHASPPACEATSPWCLFGSELPQDARVAPSTQGVLPLLMSRPEGKAGDSSGTA 177
C     APPGPERSPASPAVCEAPSPWCLFGPELVQDARAAPAAQGVLLPLMSRPESKAGDTPGAA 176
      :*.*. :* .**..**. :.*****.** :*.  .*::: :*  ****.  *.**:.*:.

H     AAHKVLPRGLSPARQLLLPASESPHWSGAPVKPSPQAAAVEVEEEDGSESEESAGPLLKG 239
M     AGQKVLPKGLSPPRQLLLPTSGSAHWPGAGVKPSPQPAAGEVEEDSGLETEGSAAPLLKS 240
P     AAHKVLSRGLAPSRQLLPSTAGSHHWPAAAVKPSPQPAVVEVEEEDDSESEGSMVPLLKG 237
C     AGQKVLPRGLAPCRQLLAPTASSHPWPAPAPRPSPQPAVVELEEDD-SDPEGSSGPPLKG 235
      *.:***.:**:* **** .:: *  *...  :****.*. *:**:.  :.* *  * **.

H     KPRALGGAAAGGGAAAVPPGAAAGGVALVPKEDSRFSAPRVALVEQDAPMAPGRSPLATT 299
M     KPRALEGTGSGGGVAANAASAAPGGVTLVPKEDSRFSAPRVS-LEQDSPIAPGRSPLATT 299
P     KPRPAGGTAAGTGTPTGAPGTAAGGVALVPKEDARFSAPRGALAEHDASGAPGRSPLATT 297
C     KARPAG------GDAISAPGTAAGGVAPVPKEDARFSAPRAAPAEHDAPAAPGRASVATA 289
      *.*.        * .  ...:*.***: *****:****** :  *:*:. ****:.:**:

H     VMDFIHVPILPLNHALLAARTRQLLEDESYDGGAGAASAFAPPRSSPCASSTPVAVGDFP 359
M     VVDFIHVPILPLNHALLAARTRQLLEGDSYDGGATAQGPFAPPRGSPSAPSPPVPCGDFP 359
P     VMDFIHVPILPLNTAFLAARTRQLLEGDNYDGGAPAVSAFAPPRGSPSAPSASVTAGDFP 357
C     VMDFIHVPILPLNSAFLAARTRQLLEGDSYDGG--AAGAFAPPRGSPSASAALGAPGDFP 347
      *:*********** *:**********.:.****  * ..*****.**.*.:.  . ****

H     DCAYPPDAEPKDDAYPLYSDFQPPALKIKEEEEG-AEASARSPRSYLVAGANPAAFPDFP 418
M     DCTYPLEGDPKEDVFPLYGDFQTPGLKIKEEEEG-ADAAVRSPRPYLSAGASSSTFPDFP 418
P     DCAYSADDEPKDDAFPLYGDFQPPALKIKEEEEG-AEAAARSPRSYLVAGANPAVFPDFP 416
C     DCAYQPDSEPKDDAFSLYGDPQPPALKIKEEEEGGAEAAARSPRPYLLAGASPAVFADFP 407
      **:*  : :**:*.:.**.* *.*.********* *:*:.****.** ***..:.*.***

H     LGPPPPLPPRATPSRPGEAAVTAAPASASVSSASSSGSTLECILYKAEGAPPQQGPFAPP 478
M     LAPAP---QRAPSSRPGEAAVAGGPSSAAVSPASSSGSALECILYKAEGAPPTQGSFAPL 475
P     LAPPS--LPPRASSRPGEASAAVAPTSASVSSASSSGSALECMLYKAEGAPPQQGPFPPP 474
C     LAPPS---------RPGEVAVPAAATGAPGSSAAVSGSALECVLYKAEGAPPQPGPFAPP 458
      *.*..         ****.:.. ..:.*. *.*: ***:***:*********  *.*.*

H     PCKAPGASGCLLPRDGLPSTSA-----SAAAAGAAPALYPALGLNGLPQLGYQAAVLKEG 533
M     PCKPPAAGSCLLPRDSLP---------AAPATAAAPAIYQPLGLNGLPQLGYQAAVLKDS 526
P     PCKPPSAGACLLPRDSLPSTSASAAAAAAAAAGAAPALYQPLGLNGLPQLGYQAAVLKEG 534
C     PCKAPAAGACLLPRDXCP---------STSAAAAAR--------------------RRG 488
      ***.*.*..******  *         ::.*:.**                      : .
```

```
H     LPQVYPPYLNYLRPDSEASQSPQYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFFKR 593
M     LPQVYPPYLNYLRPDSEASQSPQYGFDSLPQKICLICGDEASGCHYGVLTCGSCKVFFKR 586
P     LPQVYQPYLNYLRPDSEASQSPQYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFFKR 594
C     LPQVYQPYLNYLRPDSEASQSPQYSFESLPQKICLICGDEASGCHYGVLTCGSCKVFFKR 548
      ***** ******************.*:**********************************

H     AMEGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKKFNKVRVVRALDA 653
M     AMEGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKKFNKVRVMRTLDG 646
P     AMEGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKKFNKVRVMRALDA 654
C     AMEGQHNYLCAGRNDCIVDKIRRKNCPACRLRKCCQAGMVLGGRKFKKFNKVRVMRTLDA 608
      ******************************************************:*:**.

H     VALPQPVGVPNESQALSQRFTFSPGQDIQLIPPLINLLMSIEPDVIYAGHDNTKPDTSSS 713
M     VALPQSVGLPNESQALGQRITFSPNQEIQLVPPLINLLMSIEPDVVYAGHDNTKPDTSSS 706
P     VALPQSGGIPNESQALSQRITFSPNQDLQLIPPLINLLMSIEPDVVYAGHDNTKPDTSSS 714
C     VALPQPVGIPNESQALSQRITFSPSQDLQLIPPLINLLMSIEPDVVYAGHDNSKPDTSSS 668
      *****. *:*******.**:****.*::**:**************:******:*******

H     LLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSG 773
M     LLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSG 766
P     LLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSG 774
C     LLTSLNQLGERQLLSVVKWSKSLPGFRNLHIDDQITLIQYSWMSLMVFGLGWRSYKHVSG 728
      ************************************************************

H     QMLYFAPDLILNEQR--MKESSFYSLCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLN-TI 830
M     QMLYFAPDLILNEQR--MKELSFYSLCLTMWQIPQEFVKLQVTHEEFLCMKVLLLLN-TI 823
P     QMLYFAPDLILNEQR--MKESSFYSLCLTMWQIPQEFVKLQVSQEEFLCMKVLLLLN-TI 831
C     QMLYFAPDLILNESWGVQPATTRVSLCQCPRSCSLLHHIFNCYNFALLSWIKFLKCDSKV 788
      *************.       :  ***    . .  .  ::  :  :*.   :*  : .:

H     PLEGLRSQTQFEEMRSSYIRELIKAIGLRQKGVVSSSQRFYQLTKLLDNLHDL----VKQ 886
M     PLEGLRSQSQFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDSLHDL----VKQ 879
P     PLEGLRSQNQFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDL----VKQ 887
C     PLEGLRSQNQFEEMRSSYIRELIKAIGLRQKGVVPSSQRFYQLTKLLDNLHDISILLVKQ 848
      ********.************************.*************.***:    ***

H     LHLYCLNTFIQSRALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK 933
M     LHLYCLNTFIQSRTLAVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK 926
P     LHLYCLNTFIQSRALSVEFPEMMSEVIAAQLPKILAGMVKPLLFHKK 934
C     LHLYCLNTFIQSWALSVEFPEMMSEVIAAQLPKILAGMVKSLLFHKK 895
      ************ :*:************************.******
```

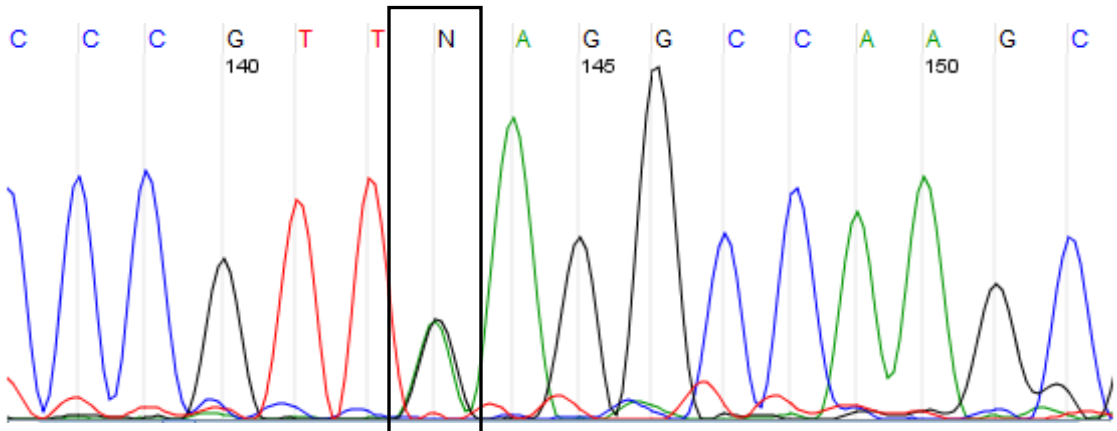
Table 3.4 Polymorphic sites in porcine PGR mRNA. Polymorphisms identified by BLASTN alignment of mRNA (GQ903679.1) sequence between individuals and with genomic (CU640601.2) sequence. Location indicates the exon containing the polymorphism with base number of GQ903679.1 sequence in parentheses. Resultant effects of nucleotide identity on amino acid sequence, if any, and position in the predicted protein are listed. Shift indicates a shift in reading frame.

Location (base)	mRNA (GQ903679.1)	Genomic (CU640401.2)	Amino acid (Residue)
Exon 1 (691)	C	T	NON-CODING
Exon 1 (954)	C	T	NON-CODING
Exon 1 (1288)	G / C	G	ARGININE TO THREONINE (87)
Exon 1 (2401)	T	Gap	SHIFT
Exon 1 (2582)	C/ T	C	LEUCINE (518)
Exon 2 (2801)	Gap	T	SHIFT
Exon 2 (2805)	T	Gap	SHIFT
Exon 7 (3513)	A	C	ASPARAGINE TO HISTIDINE (829)
Exon 7 (3519)	A	G	ISOLEUCINE TO VALINE (831)

Location (base)	mRNA (GQ903679.1)	Genomic (CU640401.2)	Amino acid
Exon 8 (3895)	A	G	NON-CODING
Exon 8 (3971)	Gap	A	NON-CODING
Exon 8 (4294)	A / C	A	NON-CODING
Exon 8 (4354)	A	G	NON-CODING
Exon 8 (4470)	T	G	NON-CODING

Figure 3.14 Chromatographs of DNA sequences containing SNP 2582 (C/T) of the progesterone receptor in individual samples of pig uterine tissue. Sequences were generated from A) and B) two individuals both heterozygous at position 2582 (GQ903679.1). The published genomic sequence (CU640401.1) contains a C residue. Sequences shown are from antisense strands generated from a reverse primer.

A



B

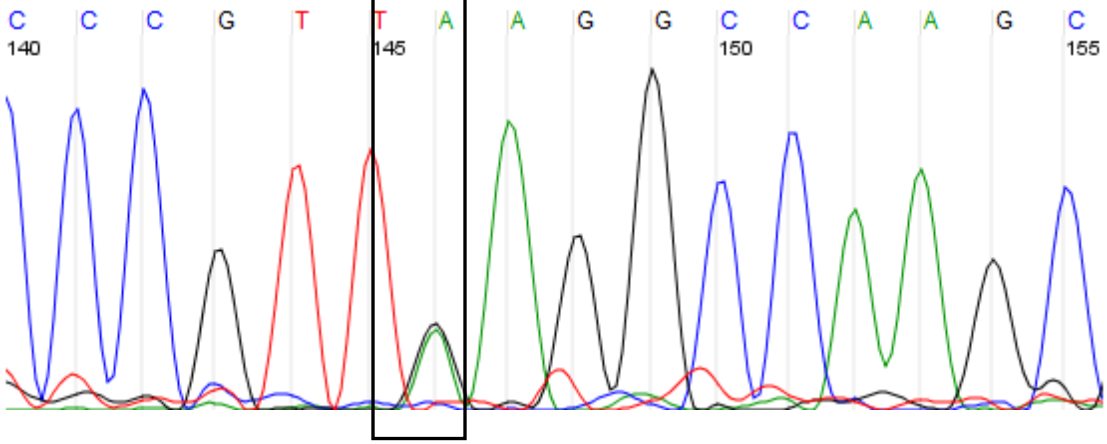
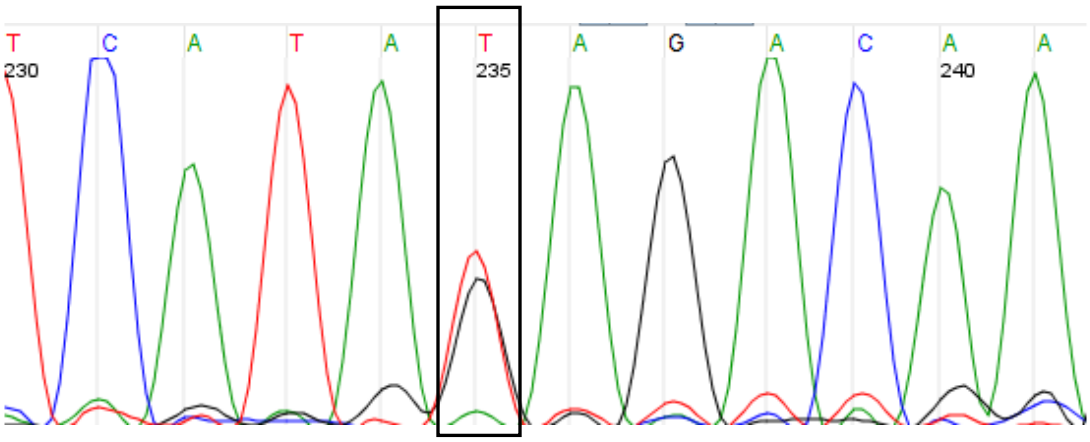


Figure 3.15 Chromatograph of DNA sequence containing SNP 4294 (A/C) of the progesterone receptor in individual samples of pig uterine tissue. Sequence was generated from a heterozygous individual at position 4294 (GQ903679.1). Sequence shown is from antisense strand generated from a reverse primer.



Another sequence from porcine uterine mRNA was submitted to GenBank in February of 2010 (NM001166488.1; Sukjumlong et al., 2010). The Sukjumlong sequence is also 4515 bases in length and is 100% identical to the PGR mRNA generated here in endometrium (GQ903679.1), validating the accuracy. Several mRNA sequences containing partial PGR confirm the sequence of GQ903679.1. An expressed sequence tag (EST) aligning to bases 2233 – 2467 is 100% identical to GQ903679.1. Another aligning to bases 2548 – 2651 is 99% identical to GQ903679.1. Testicular mRNA aligning to bases 3165 – 3808 is 99% identical (AJ245450.1) and granulosa cell mRNA aligning to bases 3459 – 3810 is 98% identical (S49016.1). Taken together, these ESTs further validate the 3' half of the PGR mRNA sequence.

DISCUSSION

Despite a manageable transcript length of 4.3 kb, we were unable to amplify full-length PGR mRNA. A guanine-cytosine rich (69.5%) region located in the 3' region of exon 1 (2172 – 2755) was difficult to amplify and could only be accomplished using a DNA polymerase enzyme optimized for sequences of high GC content. We were able to amplify PGR in 8 overlapping products, and by tiling these into a contig, were able to construct a transcript sequence that agreed with the unannotated genomic porcine sequence containing PGR. Secondary structure of the PGR mRNA molecule may be responsible for the difficulty experienced in amplifying the full-length product.

We were also initially able to amplify and sequence the putative promoter region of PGR-B upstream of the predicted transcription initiation site from cDNA with primer pair PR_UP_F6, R6. This was unexpected because this sequence is not predicted to be transcribed and thus should not be present in cDNA. After DNAse treating aliquots of the total RNA samples prior to cDNA synthesis, however, we were no longer able to amplify a PCR product using the forward primer upstream to the transcription initiation site. This result indicates that genomic contamination was responsible for the unexpected PCR product.

We were able to amplify products with the other 7 primer pairs in both DNAse treated and untreated samples. Primer pairs PR_5'_GAP_F2, R2 and PR_B_F1, R1 amplify the 5' UTR of the PGR transcript. Ability to amplify the product using cDNA synthesized from DNAse treated total RNA as a template confirmed presence of this fragment in PGR mRNA transcripts. The result indicates that the transcription initiation site for PGR-B isoform is located between bases 1 and 470 of the mRNA sequence GQ903679.1. Primer pair PR_B_F3, PR_AB_R1 spans the ATG translation start sites of PGR-B and PGR-A. Primer pair PR_AB_F1, R2 amplifies a 3' region of exon 1 common to both PGR-B and PGR-A. Primer pairs PR_Ex1_GAP_F1, R1 and PR_AB_Ex1_F2, PR_C_Ex8_R2 span exons 1 and 2 and exons 2 through 8, respectively. Primer pair PR_Ex8_GAP_F1, R2 is located at the 3' end of exon 8, extending beyond the TGA stop codon into the 3'UTR. A classical polyadenylation signal sequence was not observed before base 4515 (GQ903679.1).

The CpG island located in the 3' region exon 1 (Figure 3.1: 2172 – 2755bp) is a CCG trinucleotide repeat with no other identifiable CpG island motifs. Sequences

containing CpG islands tend to be sites for alternative methylation and important for transcriptional regulation (Haberman et al., 2007). Trinucleotide repeats have been implicated as significant players in oncogenes, being 2.5 times more prevalent in cancer related genes than in other genes. Of particular interest, a CAG repeat in androgen receptor is associated with tumors in the uterus and ovaries of women (Haberman et al., 2007). Additionally, hypermethylation of PGR-B has been observed in human uteri afflicted with endometriosis (Wu et al., 2006). The motif of a CCG trinucleotide repeat and its methylation is well-described as the cause of Fragile X Syndrome. Increased methylation of CCG on the X chromosome makes the DNA inaccessible to the nucleosome, blocking transcription (Wang and Griffith, 1996). This evidence indicates that further investigation of the CCG trinucleotide repeat in porcine PGR and variations in methylation status during the cycle or between individuals is likely to be pertinent to understanding PGR regulation.

Differences in nucleotide sequence of mRNA (GQ903679.1) and genomic (CU640401.1) sequences encode 2 non-conservative amino acid substitutions. Two of these substitutions (1288 (G/C) and 3513 (A/C)) result in dramatic changes in the chemical properties of the amino acid residue. The 1288 (G/C) SNP is a transition from a positively charged arginine residue to a polar, hydrophilic threonine residue (R87T). This SNP is located in the variable region of the PGR protein (NP001159960.1). The consequences of a non-conservative substitution in this region are unknown. The mutation may alter the conformation and folding of the N-terminus of PGR, with uncertain effects on protein function. The 3513 (A/C) SNP results in a transition from a hydrophilic, polar asparagine residue to a positively charged histidine residue (N829H).

The SNP is located in the LBD of the PGR protein, and thus may have severe consequences on the correct folding and function of PGR. A third SNP in the coding sequence, 3519 (A/G), results in a conservative amino acid substitution of hydrophobic, non-polar residues, transitioning from isoleucine to valine. Of 6 sequences containing the 1288 (G/C) polymorphism, 5 contained a guanine residue, as did the genomic sequence (CU640401.1). However, visual examination of the generated chromatographs verified a cytosine residue at this position in the sixth sample, suggesting this may be a minor allele in the population.

The three gaps in the coding region together result in a net shift of GQ903679.1 by 1 bp from the genomic sequence, completely altering the reading frame. The PGR protein predicted from the mRNA sequence was determined to be accurate by aligning the proteins predicted by the mRNA sequence and genomic sequence, and then comparing concordance of both with protein sequences from humans, mice, and cow.

In humans, 5 SNPs in the protein coding region of the PGR gene have been identified (De Vivo et al., 2002; Schweikert et al., 2004; NCBI refSNP, NCBI, Bethesda, MD). Two of these result in synonymous amino acid residue substitutions: a C to A/G/T transition at position 1922 of the human PGR mRNA sequence (NM000926.4) resulting in retention of a glycine residue at position 393 of human PGR protein sequence (NP000917.3; G393G) and H770H as a result of a C to T transition at base 3053 (De Vivo et al., 2002). Two additional polymorphisms result in non-synonymous, but conservative amino acid substitutions: S344T arising from a G to C transition at base 1031 and V660L arising from a G to T transition at base 2721. Finally, at position 3401 base the ancestral A residue was observed to transition to G, C or T (Schweikert et al.,

2004). An A to G residue transition results in a synonymous amino acid residue (Q886Q). A transition from an A residue to either C or T results in a non-conservative transition from glutamine to histidine Q886H.

None of these locations correspond to positions at which we discovered SNPs within the currently available pig sequences. The variable residues S344T and V660L, however, do align to positions at which the porcine amino acid sequence differs from the human sequence. At position 344, the residue identity in both mice and pigs is glycine, while the human sequence can be either serine or threonine. While a threonine to serine transition is a conservative substitution since they are both polar amino acids and hydrophilic, glycine is a non-conservative substitution, since it is non-polar and hydrophobic. This may suggest that there is little selective pressure to conserve the chemical disposition of the residue at this position, indicating the residue may not play an essential role in protein tertiary structure or PGR function. At position 660, the residue identity in humans, mice and cows is valine. In humans, an alternative allele results in a leucine residue being substituted for valine. In pigs, a glycine residue is located at this position. All three amino acids are non-polar and hydrophobic, suggesting that selective pressure at this location may require only inclusion of a non-polar residue, rather than a specific amino acid.

As expected, porcine PGR homology to human, mouse and bovine PGR protein was highly conserved in the DNA and hormone binding domains at the C terminus of the protein which are essential to protein function, while inter-species homology was lower in the N-terminal variable region of the protein. Likewise, at the transcript level, porcine PGR homology with human, mouse and bovine PGR was lowest in the 5’ UTR for PGR-

B and undetectable in the 5' putative promoter region, perhaps due to low sequence coverage. Like human, mouse and bovine PGR, the porcine gene had no classic TATA box in the proximal promoter region (contained within GQ903679.1) of PGR-B, nor in the putative PGR-A and PGR-C promoter regions. The porcine PGR nucleotide sequence, however, does give rise to 3 in-frame ORFs, which is supported by previous understanding of human and mouse PGR isoforms that encode identical proteins with N-terminus truncations arising from 3 separate promoters. Conservation of these sites in pig suggests the presence and potential differential regulation of these isoforms in pigs.

CONCLUSIONS

A full-length, 4.3 kb PGR mRNA sequence was built using 8 primer sets. Putative promoter region was sequenced using a genomic DNA template, and transcript sequence using a cDNA template. The PGR-B transcript initiation site was determined to be located between bases 1 and 470 of GQ903679.1 sequence. Difficulty in sequencing a full-length amplicon and regions enriched in guanine and cytosine content suggests that PGR mRNA may form a complex secondary structure. The sequence was verified by several GenBank *Sus scrofa* ESTs and by homology to porcine genomic DNA. Three start codons give rise to 3 in-frame open reading frames, predicting 3 protein isoforms with progressive N-terminus truncations (PGR-B, PGR-A and PGR-C).

The PGR mRNA sequence has high homology with human, mouse and cow PGR mRNA. At the protein level, pig PGR sequence was also well-conserved in human,

mouse and predicted cow PGR amino acid sequences. Conservation between human and pig protein was higher in the C, D, and E domains of the protein, with lower conservation of amino acid identity in the A/B variable domain. The porcine PGR mRNA derived protein sequence and genomic derived protein sequence were 98% identical. Eleven SNPs were identified between mRNA and genomic PGR sequence: 7 located in untranslated regions of the transcript, 1 resulting in synonymous amino acid residue, 1 resulting in a conservative amino acid substitution and 2 non-conservative amino acid substitutions that may affect functionality of the PGR protein encoded.

Further study of the affect of amino acid substitutions on PGR protein conformation and function and potential impacts on fertility may elucidate possible benefits or demerits of one allele over another. Analysis of additional animals may lead to the discovery of additional SNPs in the PGR gene and would facilitate calculations of allele frequency in the current pig population.

CHAPTER IV

CHARACTERIZATION OF PROGESTERONE RECEPTOR (PGR) ISOFORM MRNA EXPRESSION IN THE ENDOMETRIUM OF CYCLIC AND PREGNANT PIGS

ABSTRACT

The down-regulation of the PGR within the luminal epithelium enables conceptus attachment and signaling within the porcine uterus. In humans, three PGR mRNA isoforms (PGR-A, PGR-B, and PGR-C) arise from alternative transcription start sites. The mRNA encode proteins with different N-termini that may confer distinct biological functions. After determining the sequence of porcine PGR and its high homology to human PGR, the objective was to characterize isoform mRNA expression in endometrium during the estrous cycle and pregnancy. Primer pairs for quantitative real-time PCR (qRT-PCR) were originally developed from porcine genomic and mRNA sequence and used to amplify porcine PGR fragments from cDNA. Based on cDNA sequencing and porcine genomic sequence, the porcine PGR mRNA is 4.3 kb. RT-PCR primers for PGR-B (5'-TCAGACTGAAGTCGGGGAAC-3' and 5'-GGGTGAAATCTCCACCTCCT-3') and PGR-AB (region common to both PGR-A and PGR-B; 5'-GCTCCATGGTTCCACTTCTG-3' and 5'-GATGGGCACGTGGATAAAAT-3') were developed to study PGR regulation in

endometrial tissue from cyclic (days 0, 5, 7.5, 10, 12, 13, 15, 17) and pregnant (days 10, 12, 13, 15, 17) pigs (n = 53 samples; minimum of 4 pigs per status per day). There was a tendency for an effect of d on uterine PGR-B expression ($P < 0.10$), because PGR-B mRNA fold change was greater on day 0 (d 0; 0.52 ± 0.07) and d 5 (0.51 ± 0.07) compared with d 7.5 (0.31 ± 0.07) and d 15 (0.30 ± 0.05) (remaining d were intermediate). The PGR-AB mRNA remained low through d 13 (0.13 ± 0.01; d 0 to 13; cyclic and pregnant) and increased on d 15 in both pregnant (0.90 ± 0.07) and cyclic (0.41 ± 0.07) pigs ($P < 0.001$). The PGR-AB mRNA remained elevated in pregnant pigs on d 17 (0.33 ± 0.06). The existence of PGR-B and possibly PGR-A isoforms were detected by Northern Blot analysis. PGR-C, however, was not detected. Transcription initiation sites were detected in PGR by RPA: *PGR-B.1*, 291-314; *PGR-B.2*, 379; *PGR-A*, 1046; *PGR-C.1*, 2559; *PGR-C.2*, 2631. We conclude that PGR isoform mRNA abundance may change during the estrous cycle and pregnancy potentially leading to functional differences in PGR action. We also observed the presence of PGR-B, PGR-A and PGR-C in qRT-PCR and ribonuclease protection assay analyses, although some transcripts may be too unstable to be consistently detected by Northern blot analysis.

INTRODUCTION

Ability of a developing conceptus to develop and signal its presence to the maternal environment is a crucial and precarious event in establishing pregnancy.

Porcine embryonic loss by day 9 post conception has been estimated to be 21.4% (Scofield et al., 1974). Prenatal mortality in the pig has been estimated to range from 20% (Perry, 1954) to 50% (Hanley, 1961) with the majority of loss occurring between days 10 to 20 of gestation. The period of embryonic loss is during a critical time for conceptus development in the pig because the tubular embryo undergoes rapid elongation to a filamentous form within 2 to 3 hours (Geisert et al., 1982a). Conceptus attachment to the endometrium depends on the down-regulation of PGR within the uterine LE.

The uterus must undergo dynamic changes during both the establishment of pregnancy and during the estrous cycle, both in proliferation and differentiation of cells into deciduas (Larsen et al., 2002). In both uterine states, high, sustained concentrations of progesterone (P4) stimulate the rapid removal of progesterone receptor (PGR) from the uterine luminal epithelium (LE; Tseng and Zhu, 1997; Harduf et al., 2009; Kurita et al., 2000a, 2001; Mote et al., 1999; Arnett-Mansfeld et al., 2004). This ligand induced down-regulation of PGR is an essential event for return to cyclicity in non-pregnant gilts and sows and permits attachment of the conceptuses in pregnant animals. Failure to achieve down-regulation of PGR could have severe repercussions, particularly in maintenance of pregnancy.

Previous studies in other species, like sheep, have demonstrated the disappearance of PGR from the luminal epithelium in both the estrous cycle and in early pregnancy, as well (Spencer and Bazer, 1995). Geisert et al. (1994) demonstrated using IHC that PGR protein was present in high concentration from day 0 to day 5 of the estrous cycle, but that the presence rapidly decreased by day 10 and was undetectable by day 12. No recovery in PGR protein was seen by day 18. A similar pattern was observed in pregnant

gilts. A parallel study using immunohistochemistry yielded comparable results, with PGR being down regulated in the luminal epithelium of both pregnant and cyclic gilts from days 10 to 18 (Sukjumlong et al., 2005).

Finally, *in situ* hybridization with a PGR-specific cRNA probe was used to localize PGR transcript expression at similar time points during the estrous cycle and early pregnancy (Ross et al., 2010). The results again demonstrated the presence of PGR transcripts from days 0 to 5, with rapid loss in both pregnant and cyclic gilts until complete depletion by day 13. Concordantly, Spencer and Bazer (2002) reported that PGR in the luminal epithelium of sheep is also undetectable by day 11, with subsequent disappearance from the glandular epithelium by day 13.

These results indicate a conserved pattern of PGR expression at both the mRNA and protein level during both the estrous cycle and early pregnancy. However, none of these results differentiate between variant PGR isoforms, which may be regulated independently, have different signaling pathways and confer distinct biological functions. The purpose of this study, therefore, was to differentiate between the PGR mRNA isoforms and their potentially independent expression patterns during the estrous cycle and early pregnancy in different cell types of the porcine uterus.

MATERIALS AND METHODS

Northern Blot with DNA Probe

Samples

Endometrial total RNA was isolated as described in Chapter 3 from University of Missouri – Columbia collected tissues from 8 representative endometrial samples collected from 8 individual animals.

cDNA cloning

Sequence for each of 5 primer pairs (Table 4.1) spanning exons 1-6 of porcine PGR was amplified from porcine endometrium cDNA as described in Chapter 3 using Platimun® Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The 5 products were B1 (197 bp), B2 (182 bp), AB1 (232 bp), AB2 (82 bp), and ABC (208 bp). The thermal cycler program for Platimun® Taq DNA Polymerase was: initial denaturation at 94°C for 2 minutes, thirty-five cycles with melting at 94°C for 15 seconds, annealing at 58°C for 35 seconds, and extension 72°C for 60 seconds, with a final extension cycle of 65°C for 10 minutes. Amplified products were then purified using Qiaquick PCR Purification Microcentrifuge Kit (Quiagen Inc., Valencia, CA) using the microcentrifuge protocol according to manufacturer's recommendations. Purified products were then cloned into One Shot® Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA) using TOPO TA Cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations.

Briefly, 4 µL of fresh PCR product was combined with 1 µL each of salt solution and TOPO pCR®4 vector. Mixture was incubated for 5 minutes before transformation into E. coli cells thawed on ice. Then 2 µL of the TOPO cloning reaction was added to the vial of cells and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds at 42°C and transferred to ice. Cells were then incubated in 250 µL of SOC medium for 1 hour at 37°C while shaking. After incubation, 40 – 50 µL of transformed cells were inoculated onto prewarmed selective plates (100 µg/mL ampicilin LB agar plates with 40 µL of [20 µg/mL] X-gal spread over the surface) and incubated overnight at 37°C.

Dual screening with ampicilin and X-gal resulted in the growth of white colonies putatively positive for the gene insert. A sample of these colonies were picked and grown in 3 mL of 50 µg/mL LB broth overnight in a shaking 37°C incubator. Mini-preps were used to purify 2 µL of these samples using QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA). Purified plasmid was then digested with EcoR1 enzyme and run on a 1% agarose gel next to undigested plasmid to assess the presence of an insert. If an insert of the correct size was present, M13 primers (F: 5' CTGGCCGTCGTTTTAC 3'; R: 5' GTCATAGCTGTTTCCTG 3') were used to sequence the plasmids at the University of Missouri – Columbia DNA Core Facility. Sequences were analyzed as described in Chapter 3 to validate the identity of the plasmid insert. Large preparations of positive plasmids were then grown in 25 mLs LB plus 50 µL/mg ampicilin cultures. The QIAprep Midiprep Kit (Qiagen Inc., Valencia, CA) was used to purify the plasmid DNA according to manufacturer's specifications.

DNA probe synthesis

The DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Indianapolis, IN) was used in accordance with the manufacturer's protocol to label cDNA probes. The probes were synthesized from PCR amplification products of cDNA clones. Products of PCR were purified using Qiaquick PCR Purification Microcentrifuge Kit (Quiagen Inc., Valencia, CA). In short, PCR products were column purified with PBI and PE buffers and then eluted in ddH_20. The PCR products amplified by 5 primer pairs were pooled to create a comprehensive PGR DNA probe spanning exons 1-6 for Northern blotting (Table 4.1). Three µg of pooled DNA template were denatured for 10 minutes in a boiling water bath and immediately chilled on ice. Then 4 µg of DIG-High Prime buffer containing random hexamers and Klenow enzyme was added for random integration of alkali-labile digoxigenin-dUTP residues, included in the dNTP mix. The reaction was allowed to proceed for 20 hours before 2 µL of 0.2 M EDTA was added to the reaction and heated for 10 minutes at 65°C to stop the labeling reaction.

Northern Blot

The DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Indianapolis, IN) was optimized for laboratory conditions. Ten µg total RNA in glyoxyl and DMSO buffer was incubated for 1 hour in a 50°C water bath to denature mRNA secondary structure and samples transferred immediately into an ice bath to cool. Four µL aliquots of the samples were then size separated by electrophoresis on a 1.0% agarose gel overnight with circulating 10 mM sodium phosphate buffer. The progress of

the size separation was estimated by position of the blue loading dye in the gel. Once bands were detected approximately 8 mm from the well, electrophoresis was halted and the first two lanes of the gel containing pooled total RNA and a blank well were removed, stained with ethidium bromide and visualized and photographed under ultraviolet light alongside a ruler to denote the porcine 28S and 18S rRNA bands of known size. Location of 28S and 18S rRNA bands relative to position in mm from the well was used to calculate the conversion factor for physical distance of the sample bands from the well to molecular weight of the molecules. The remaining lanes of the gel were placed on a 1 cm stack of 3MM paper soaked in 10X SSC. A nylon membrane was placed on top of the gel and covered with 2 additional pieces of 3MM wicking paper followed by 4 cm of paper towels. Finally, a glassware dish was placed on top of the paper towels to evenly distribute weight across the surface of the nylon membrane for the duration of the overnight RNA transfer from the gel to the positively charged nylon membrane.

The nylon membrane was then removed and wells marked with a pen. The membrane was first placed in a UV Crosslinker (Fischer Scientific, Pittsburgh, PA) set to "optimal crosslink" and then baked in a conventional oven for 30 minutes at 93.3°C to ensure fixation of the RNA to the membrane. The membrane was pre-hybridized at 42°C for 1 hour in 15 mL DIG Easy Hyb reagent with 100 µL salmon sperm DNA to decrease non-specific binding of the probe and gently shaken. The membrane was hybridized with 5.25 mL DIG Easy Hyb and 131.25 ng digoxigenin labeled probe for 4 – 6 hours in a Kapak Sealable pouches (Kapak Corp., Minneapolis, MN) on a shaker at 42°C. The membrane was removed from the chamber and washed thoroughly in buffers of

decreasing SSC and SDS concentrations for 40 minutes. Membrane was incubated for 30 minutes with DIG Easy Hyb Blocking Solution to decrease non-specific binding of the probe to the membrane. The membrane was then incubated for 30 minutes in DIG Easy Hyb Anti-DIG Solution. The membrane was washed thoroughly again in buffers of decreasing SSC and SDS concentrations for 40 minutes, followed by a 5 minute incubation in 50 mL of DIG Easy Hyb Detection Buffer containing of an anti-digoxigenin antibody with alkaline phosphatase tag. Finally, the membrane was exposed for 5 minutes to 1 mL of the alkaline phosphatase substrate CSPD (Roche Applied Science, Indianapolis, IN) and exposed to BIOMAX™ MS film (Eastman Kodak Co., Rochester, NY) to visualize chemiluminescence of the PGR transcript.

Northern Blot with Riboprobe

Samples

Northern blots with riboprobes were conducted on 6 biological samples. Uterine tissues were collected from gilts at the University of Missouri – Columbia and processed as described in Chapter 3. Total RNA was isolated using the TRIZOL reagent (Invitrogen, Carlsbad, CA) protocol and DNAse treated as described in Chapter 3. Three of the samples were collected from endometrium on d 0 of the estrous cycle. Three of the samples were collected from endometrium on d 12 of the estrous cycle.

Poly(A) RNA isolation

To isolate polyadenylated mRNA from total RNA samples, the MicroPoly(A)Purist™ and Poly(A)Purist™ kits were used according to manufacturer's suggestions (Ambion Inc., Austin, TX). Briefly, 50 µg of total RNA was added to a tube

containing oligo (dT) cellulose to bind polyadenylated mRNA. The cellulose-bound mRNA was then washed with several buffers and column purified. Poly(A) RNA was then eluted in warm RNA Storage Solution (Ambion Inc., Austin, TX). Enriched poly(A) RNA was quantified by spectrophotometry and quality was assessed by both 260:280 absorbance (NanoDrop-1000, NanoDrop Technologies, Wilmington, DE). Visualization of 18S and 28S ribosomal RNA bands from total RNA and poly(A) RNA size separated by electrophoresis on an 0.8% agarose gel to confirm efficacy of the poly(A) enrichment procedure.

cDNA cloning

Probes were designed to span the putative transcription initiation sites of PGR-B, PGR-A and PGR-C isoforms. Primers were designed from the *Sus scrofa* PGR genomic sequence (CU640401.2) using Primer3 software as described in Chapter 3. Complementary DNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA for PGR-B (657 bp), PGR-AB (583 bp) was amplified using GoTaq Flexi® DNA Polymerase (Promega, Madison, WI) and Jumpstart Taq (Sigma, St. Louis, MO) with 2.5 % DMSO for the GC-rich PGR-C (583) fragment. Reactions were prepared according to manufacturer's protocols. The thermal cycler program for GoTaq Flexi® DNA Polymerase was: initial denaturation at 95°C for 2 minutes, thirty-five cycles with melting at 95°C for 15 seconds, annealing at 58°C for 20 seconds, and extension 72°C for 60 seconds, with a final extension cycle of 65°C for 10 minutes. The thermal cycler program for Jumpstart Taq was: initial denaturation at 94°C for 2 minutes, thirty-five cycles with melting at

94°C for 30 seconds, annealing at 58°C for 45 seconds, and extension 72°C for 60 seconds, with a final extension cycle of 72°C for 5 minutes. Product sizes were verified by gel electrophoresis on a 1.5% agarose gel.

Fragments for PGR-B, PGR-A and PGR-C were each cloned into pCR®2.1-TOPO® Vector using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA). Ten µg of each plasmid were digested with EcoR1 and gel purified. The pCR®4-TOPO® Vector (Invitrogen, Carlsbad, CA) was also cut with EcoR1 and 0.25 µl (25 ng) was mixed with 3.75 µl of the EcoR1 cut inserts. The mixtures were incubated at 55°C for 10 minutes and then cooled to room temperature. Five µL of 2X Rapid Digestion Buffer (Promega, Madison, WI) and 1 µl T4 DNA Ligase (Promega, Madison, WI) was added, incubated at room temperature for 5 minutes, and then stored on ice.

The ligations were then transformed into One Shot® E. coli cells using the One Shot® Chemical Transformation protocol (Invitrogen, Carlsbad, CA) described in Chapter 3. Transformants containing the PGR-C insert were grown at lower culture temperatures (28-30°C) for insert stability due to its high GC content. Plasmids were isolated and inserts sequenced by the University of Missouri – Columbia DNA Core Facility as described in Chapter 3.

Riboprobe synthesis

Antisense RNA probes were synthesized using the MaxiScript® T3/T7 In Vitro Transcription Kit (Ambion Inc., Austin, TX) in accordance with the manufacturer's protocol. Probes were designed to span the transcription initiation sites of PGR-B, PGR-A and PGR-C isoforms (Table 4.2). Complementary DNA clones of PGR-B, PGR-A and

PGR-C were constructed as described above. Ten µg of PGR-B, PGR-A and PGR-C plasmid DNA was linearized by overnight (15.5 hours) restriction enzyme digestion with Not 1, Xba 1, Not 1 respectively. Samples were size separated on an agarose gel by electrophoresis alongside uncut aliquots of plasmid DNA to determine complete restriction enzyme digestion and linearization of DNA. The plasmid DNA was then re-extracted from samples by phenol chloroform extraction to concentrate the DNA and remove remaining enzyme.

The T3 RNA polymerase was used to transcribe antisense RNA for the PGR-B, PGR-A and PGR-C probes. The T7 polymerase was used to transcribe RNA Century™ Marker Template (Ambion, Inc., Austin, TX) producing RNA molecules of known sizes for use as a molecular weight ladder to determine sample band size. Reactions consisting of 500 ng of linearized, concentrated DNA added to 10X Transcription Buffer, 10 mM each of unlabeled nucleotides, T3 or T7 enzyme and P^{32}-labeled CTP were incubated for 1 hour at 37°C. Riboprobes were purified and concentrated by phenol-chloroform extraction and any unbound ^{32}P-CTP removed using Quick Spin™ (TE) Sephadex® G-25 Columns (Boehringer Mannheim Corp., Indianapolis, IN) in accordance with the manufacturer's protocol.

Northern Blot

The protocol for a Northern Blot with riboprobe was adapted from Lucy (1993). Twenty µg total RNA or 2 µg enriched poly(A) mRNA was mixed with 40 µL 6M glyoxyl, 120 µL DMSO, and 24µL 100 mM sodium phosphate were denatured for 1 hour at 50°C. A marker with known molecular weight RNA transcripts (RiboRuler™ High

Range RNA Ladder, Fermentas, Glen Burnie, MD) was loaded into the first well followed by an empty well. A glycerol buffer containing bromophenol blue was added to each sample for visual assessment of gel migration and then loaded into the remaining wells of a 1% agarose gel with circulating 10 mM sodium phosphate at 100V for 3 hours. The first two rows (RNA ladder and empty well) were removed from the gel, stained in a 2.5N sodium hydroxyide and ethidium bromide solution, then destained in a sodium acetate, Tris-HCL solution. The ladder was then visualized and photographed under UV light with a ruler to determine the conversion factor between molecular weight and distance migrated on the gel.

The remaining lanes of the gel were placed on a 1 cm stack of 3MM paper soaked in 20X SSC. Protran Nitrocellulose Blotting Membrane (MIDSCI, St. Louis, MO) was placed on top of the gel and covered with 2 additional pieces of 3MM wicking paper followed by 4 cm of paper towels. Finally, a glassware dish was placed on top of the paper towels to even distribute weight across the surface of the nitrocellulose membrane throughout the overnight RNA transfer from the gel to the nitrocellulose membrane.

The nitrocellulose membrane was then removed and wells marked with a pen. The membrane was first placed in a UV Crosslinker (Fischer Scientific, Pittsburgh, PA) set to “optimal crosslink” and then baked in a conventional oven for 2 hours at 93.3°C. The nitrocellulose blot was then placed in Kapak Sealable pouches (Kapak Corp., Minneapolis, MN) with 50 mL of pre-hybridization solution containing 1 mL of [10 mg/mL] tRNA to inhibit non-specific binding and incubated at 42°C for 1 hour on a shaker. Pre-hybridization solution was removed and replaced with riboprobes (either B or A). The bags were resealed and incubated at 42°C with shaking overnight. The

nitrocellulose membrane was removed and washed with 1X SSC, 0.1% SDS solution twice for 15 minutes each at 50°C with shaking and then with 0.1X SSC, 0.1% SDS solution once for five minutes at 50°C with shaking. The membrane was washed once more with 1X SSC, 0.1% SDS solution for 10 minutes and then for 20 minutes at 65°C to remove any un-hybridized probes. The nitrocellulose blot was allowed to dry for an hour and autoradiography was performed using Classic Blue Film BX (MIDSCI, St. Louis, MO) with intensifying screens at -80°C for approximately 13 hours. The sizes of the transcript bands were then identified.

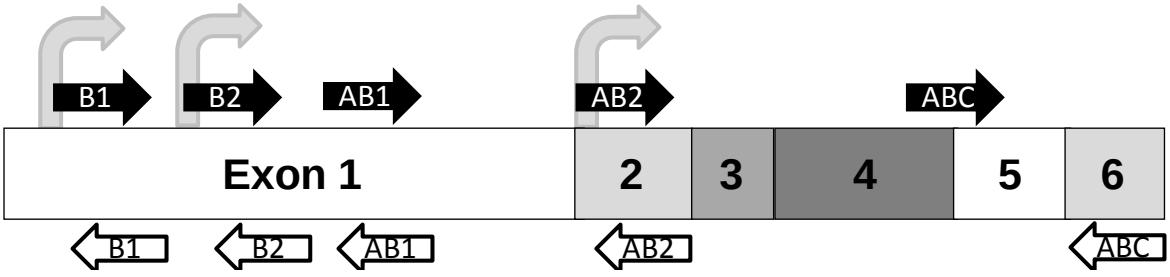
Ribonuclease Protection Assay

RPA

Ribonuclease protection assays were performed using the RPA III™ Kit (Ambion, Inc., Austin, TX). Samples of poly(A) RNA were isolated and riboprobes synthesized as described above in the Northern Blot with Riboprobe protocol. Riboprobes were designed to span the transcription initiation sites of PGR-B, PGR-A and PGR-C isoforms (Figure 4.1), with β-actin serving as a negative control. Samples and negative controls were prepared as follows: 20 µg RNA was added to 2 µL labeled probe, 2.2 µL 5M sodium acetate, and 55 µL 100% ethanol; negative controls were prepared by adding 20 µg of yeast RNA to 2 µL labeled probe, 0.6µL 5M sodium acetate and 15 µL 100% ethanol. Positive controls were prepared by adding 2 µL labeled probe to 50 µL Loading Buffer 2. All samples were incubated for 3 minutes at 90 - 95°C. One µL each of positive control samples and markers were added and 5 µL of each sample and negative control were loaded onto an 6% acrylamide, 6 M Urea gel (6% PAGE/Urea

Solution, Ambion) and size separated by electropholesis for 1 hour at 250 V and then dried for 2 hours at 80°C using a Drygel Sr. (Hoefer Scientific Instruments, San Francisco, CA). Once dry, autoradiography was performed using Classic Blue Film BX (MIDSCI, St. Louis, MO) or BIOMAX™ MS film (Eastman Kodak Co., Rochester, NY) with intensifying screens at 25°C. Protected fragments were then identified using RNA Century™ Marker Template (Ambion, Inc., Austin, TX) for size comparison.

Figure 4.1 Model representation of genomic locations of the 5 primer pairs used for qRT-PCR (B1, B2, AB1, AB2 and ABC). Exons 1 through 6 containing primer pairs are shown (exon 7 and 8 omitted). Forward primers are represented by black arrows. Reverse primers are represented by white arrows. Approximate transcription initiation sites are represented by curved gray arrow for PGR-B, PGR-A and PGR-C transcripts, respectively. Primer pairs B1, B2 and AB1 are all located within exon1. Primer pair AB2 is located within exon 2. The forward primer of the ABC pair spans exons 4 and 5, while the reverse ABC primer is located in exon 6. The B1 primer set is capable of detecting only the full-length PGR-B transcript. Both the B2 and AB1 primer pairs detect both the PGR-B and PGR-A transcripts. The AB2 and ABC primer pairs detect the PGR-B, PGR-A and PGR-C transcripts.



Real-time PCR

Samples

Uteri from cyclic (d 0, 5, 7.5, 10, 12, 13, 15, 17) and pregnant (d 10, 12, 13, 15, 17) gilts was collected by hysterectomy (n = 53) at Oklahoma State University. Endometrium (5-10 g) was manually extracted from the myometrium using sterile scissors, flash frozen in liquid nitrogen and stored at -80°C (Ross et al., 2010).

RNA isolation

Total cellular RNA was isolated from approximately 500 mg of endometrium using RNAwiz (Ambion, Austin, TX) in accordance with the Ambion protocol at Oklahoma State University. Pellets were resuspended in ddH_20 (Ross et al., 2010). Samples were transported to the University of Missouri-Columbia on dry ice and stored at -80°C. Isolated RNA was quantified at the University of Missouri – Columbia by spectrophotometry (NanoDrop-1000, NanoDrop Technologies, Wilmington, DE). Sample integrity was assessed both by NanoDrop 260:280 nm absorbance ratio and also in aliquots of 500 ng per sample with bromophenol blue loading buffer by visualization of 28S and 18S ribosomal RNA on a 0.8% agarose gel (with 0.09 M Tris-borate, 0.002 M EDTA buffer and 0.5 µg/mL ethidium bromide). An aliquot of 5 µg of each total RNA sample was treated with DNAse I enzyme (Ambion Inc, Austin, TX) and incubated for an hour at 37°C. The DNAse efficacy was confirmed visually on a 0.8% agarose gel.

cDNA synthesis

Two samples of cDNA were synthesized from each biological replicate: one from an aliquot of untreated total RNA and one from an aliquot of DNAse treated total RNA. Five µg of total RNA or DNAsed total RNA from each sample was used for cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with random hexamer priming in a 100 µL reaction in accordance with manufacturer's protocol. Quantity yield of cDNA was assessed by spectrophotometry and quality was assessed both by 260:280 nm absorbance ratio (NanoDrop-1000, NanoDrop Technologies, Wilmington, DE) and visualized on a 1.2% agarose gel.

Primer design

A reference sequence was generated from human PGR mRNA (NM000926.4) alignment with genomic porcine SSA 9 DNA (CU640401.2) as described in Chapter III. Five oligonucleotide primer pairs were then designed using publicly available Primer3 software v 0.4.0 (Primer3, Totowa, NJ) for qRT-PCR analysis (Table 4.1). Three primers were originally designed: one to assay PGR-B (amplifies sequence specific to PGR-B) located in exon 1 (B2), one to assay PGR-AB (amplifies sequence common to PGR-B and PGR-A) located in exon 2 (AB2), and one to assay PGR-ABC or total PGR which spans exons 4 to 6 (amplifies sequence common to PGR-B, PGR-A and PGR-C). While B2 and AB2 were located 5' to the translation start sites of PGR-A and PGR-C, respectively, further comparison to human PGR sequence suggested that these primers may also amplify the 5' UTR of PGR-A and PGR-C transcripts, respectively. The B1 primer pair located in the 5' region of exon 1 and AB1 primer pair spanning exons 1 and

Table 4.1 Primer names, sequences and locations used for Northern DNA probe and qRT-PCR amplification. Exon location and base position in PGR (GQ903679.1) gene are shown. Primers listed top to bottom in 5' to 3' orientation. The primer pair designed to YWHAG (CO945226) used for qRT-PCR control.

Primer Name	Sequence	Exon (Base Location)
B1 Forward	TCAGACTGAAGTCGGGGAAC	Exon 1 (692 – 711)
B1 Reverse	GGGTGAAATCTCCACCTCCT	Exon 1 (870 – 889)
B2 Forward	CCTGGACGGGCTACTCTTC	Exon 1 (1187 – 1205)
B2 Reverse	TCCAAGACACTGTCCAGCAG	Exon 1 (1350 – 1369)
AB1 Forward	GCTCCATGGTTCCACTTCTG	Exon 1 (1714 – 1733)
AB1 Reverse	GATGGGCACGTGGATAAAAT	Exon 1 (1927 – 1946)
AB2 Forward	GATTCAGAAGCCAGCCAGAG	Exon 2 (2673 – 2692)
AB2 Reverse	GATGCTTCATCCCCACAGAT	Exon 2 (2736 – 2755)
ABC Forward	AAGTCACTGCCAGGTTTTCG	Exon 4 (3231 – 3250)
ABC Reverse	TGCCACATGGTAAGGCATAA	Exon 6 (3420 – 3439)
YWHAG Forward	TGCTAAGAATGGTCCGTGTG	
YWHAG Reverse	GGGAGCAAAAGAAACACCAG	

2 were designed to ensure exclusion of the 5' UTR of other isoforms and increase confidence in the specificity of the isoform detection of PGR-B only and PGR-AB only (Figure 4.2; Table 4.2).

Tyrosine-3-monooxygenase/tryptophan 5 monooxygenase activation protein gamma polypeptide (YWHAG; CO945226.1) was used as a control gene, as it has previously been demonstrated to be consistent across stages of cyclicity and pregnancy in the porcine uterus via both microarray and qRT-PCR experiments (Kim, 2007). The YWHAG transcript was also demonstrated to be an adequate "housekeeping" gene during stages of porcine embryo development (Whitworth et al., 2005).

Quantitative Real-Time PCR (qRT-PCR)

The primers were designed as described above (Table 4.1) and cDNA was amplified by PCR. The size of the PCR product was determined by gel electrophoresis (1.2% agarose). Products of the expected size were then sequenced in both directions using either the forward or reverse primer from PCR amplification on a 3730 96-capillary DNA Analyzer using the Big Dye Terminator (Applied Biosystems, Foster City, CA) method at the University of Missouri – Columbia DNA Core Facility. Chromatographs were visually assessed for call rate and any additional unambiguous base detection. The sequences were then aligned against human PGR mRNA (NM000926.4) and porcine genomic PGR (CU640401.1) or the control gene YWHAG (CO945226.1) sequence to verify the identity of the product amplified.

Semi-quantitative real–time polymerase chain reaction (qRT-PCR) was then performed by an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City,

Figure 4.2 Location of 3 riboprobes designed for Northern blot RPA analyses and position relative to translation start codons of PGR-B, PGR-A and PGR-C. Riboprobes used to detect the transcription initiation sites are in bold and underscored with a dotted line, spanning, from 5' to 3', PGR-B (nucleotides 1 – 470), PGR-A (901 – 1147) and PGR-C (2172 – 2755) isoform predicted initiation sites. Probes were designed using the known locations of PGR-B, PGR-A and PGR-C isoforms in human. Translation start codons (ATG) for PGR-B, PGR-A and PGR-C isoforms, respectively, are represented in bold with double underline.

```
   1 ttcttcttcc agggaaaaat ctttacaaaa ttgaaatctt gaaaattcag cttctgatag
  61 aatccattcg cttttccagt tagactgggc ataattctta attggtagta cacaatctaa
 121 tatgcttacg aagttccatc ctcacagtac ctgtttttta gactagcggt tgggaatact
 181 aactccagaa tttcagatct ggtccataat tggggcaggg aggggctttg ggcggagcct
 241 ccggataggg aggaggcatt gttagaaagc tgtggggcta gtctactggc tgtcactact
 301 ctagttaagc ctttgtattt gaatgtgcga ggagggtctg ctgtgttagc agcattttca
 361 gtgagaacta gtctcacttg gcatttgagt ggaatctgca cctacaggcg tttcgtcccc
 421 tccggactgc taagaaatgt gagggctgcg gagatgactg tcgccagcta tagggaaccg
 481 ggaggtataa gactgttcag ggaatgggct gtgccgagag gccaaaatag ccccagggtt
 541 ttagggagac gggaaatgta acacaggcag ggactggcag caacagggca ttgaagccgg
 601 agaaaaagtc gggagataga gccgccagta attgctttcg aagccgccgc cactcgagtg
 661 ctgacttgtg agtactctgc gtctcctgtc ttcagactga agtcggggaa ctctcttgga
 721 gaactctccc agttaggaaa cgagatccct acaactacct tcgccggctc tccccacctg
 781 cctcaaggac gtaacaacta tcacccatct cctgcctacc agacgctggg caagggcagg
 841 agctgacagg tgccgccccc ttccgaccca ggaggtggag atttcacccc cctctccacc
 901 gtcccgtcca gccaaattca acacctattt tctcctcccg ctgcccctat attcccgaca
 961 ccccctcatc cttcccctat ccctcctccc cagagacagg ggaggagaaa aggggagtcc
1021 aggtcgtcat gactgagctg aaggcaaagg atccccgggc tccccacgtg gcgggcagag
1081 cgccctcccc cacccagctc gggacgctgg gacgcccaga cacaggcccc tttcaggcga
1141 gccagacctc ggaagcgtcg cccgcagcct cggccatacc cctctccctg gacgggctac
1201 tcttccctgg gccctgccag ggacaggaac cagacgggaa gacgcaggac cagcagtcgc
1261 tgtcagacgt ggaggggcgg tatcccacag ttgaagctac agagggtgct ggaggtggca
1321 gctctagacc ctcggaaaaa gacaccgggc tgctggacag tgtcttggac acgctactag
1381 cgccctcagg tcccgggcag agccacgcca gccctcccgc ctgcgaagcc accagccctt
1441 ggtgcttgtt tggctctgag cttccccagg acgctcgggt tgcccttcc acccagggag
1501 tattgccccc gctcatgagc cggccagagg gcaaggctgg cgacagctcc gggacggcag
1561 ccgcccataa agtgctatcc aggggtctgg caccgtcccg gcagctgctg ccctcgactg
1621 ccgggagcca tcactggccg gcggccgcag tgaagccctc tccgcagccc gccgtggtgg
1681 aggtggagga ggaggatgac tccgagtccg agggctccat ggttccactt ctgaagggta
1741 aaccccggcc tgcaggaggc acggctgccg gaacaggaac cccgaccggt gctcctggga
1801 cggccgcagg aggcgtcgcc ctggtcccca aggaagatgc ccgcttctcg gcgcccaggg
1861 gcgccctggc ggagcacgat gcgtcagggg cgcccgggcg ctcccctctg gccactacgg
1921 tgatggattt tatccacgtg cccatcctgc cgctcaacac ggcctttctg gccgcccgca
1981 cccggcaact gctagagggg gataactatg acggcggggc cccggctgtc agcgcctttg
2041 caccgccgcg gggctcgccc tcggccccgt ccgcctccgt caccgccggc gacttccccg
2101 actgcgcgta ctctgcagac gacgagccca aggacgacgc attcccgctc tatggcgact
2161 tccagccgcc cgccctgaag atcaaggaag aggaggaagg cgccgaggcc gccgcgcgct
2221 ctccgcggtc gtacctggtg gcgggtgcca accctgccgt cttcccggat tttccgctgg
2281 cgccgccgtc gctgcctccg cgagcgtcgt ccagacctgg ggaagcctcg gcggcggtcg
2341 cacccaccag tgcctcggta tcgtcggcgt cctcgtcggg gtcggccttg gagtgcatgt
2401 tgtataaggc ggagggcgcg ccgccccaac agggcccgtt cccgcctccg ccctgcaagc
2461 cgccgagcgc cggcgcctgc ctgctgccgc gggacagcct gccgtccacc tcggcctccg
2521 ccgccgccgc cgccgccgcc gccgccgggg cggctcctgc gctctaccag ccgcttggcc
2581 ttaacgggct cccgcagctc ggctaccagg ccgcggtact caaggagggc ctgccgcagg
2641 tgtaccagcc ctatctcaac tacctgaggc cggattcaga agccagccag agcccacagt
2701 acagcttcga gtcattacct cagaagattt gtttaatctg tggggatgaa gcatcaggct
2761 gtcattatgg tgtccttacc tgtgggagct gtaaggtctt ctttaaaagg gcaatggaag
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Table 4.2 Primer names, sequences and locations used to synthesize probes from porcine cDNA clones for Northern Blot and RPA analyses. Exon location and base position in PGR (GQ903679.1) gene are shown. For β-actin primers, exon location and base position in (U07786) gene are shown. The PGR-B transcription initiation site is spanned by PR_B_F and PR_B_R. The PGR-A transcription initiation site is spanned by PR_AB_F and PR_AB_R. The PGR-C transcription initiation site is spanned by PR_GC_F and PR_GC_R.

Primer Name	Sequence	Exon (Base location)
PR_B_F	TCTTCTTCCAGGGAAAAATCT	Exon 1 (1 - 21)
PR_B_R	TAGCTGGCGACAGTCATCTC	Exon 1 (451 - 470)
PR_AB_F	GTCCAGCCAAATTCAACACC	Exon 1 (906 - 925)
PR_AB_R	GTCTGGCTCGCCTGAAAG	Exon 1 (1130 - 1147)
PR_GC_F	GCCCTGAAGATCAAGGAAGA	Exon 1 (2172 - 2191)
PR_GC_R	GATGCTTCATCCCCACAGAT	Exon 2 (2736 - 2755)

CA), using the Power SYBR® Green protocol (Applied Biosystems, Foster City, CA). Reactions were prepared in a 25 µL volume containing 100 ng of cDNA and a 1 µM concentration of both forward and reverse primers. Reactions were amplified using 40 cycles of a 2-stage program of melting for 15 seconds at 96°C and annealing and extension for 1 minute at 60°C after an initial incubation for 2 minutes at 50°C and 10 minutes at 95°C, the optimal polymerase activation temperature. Sequence Detection Software (Applied Biosystems, Foster City, CA) was used for recording and analysis of amplification plots to determine the cycle in which the product abundance reaches a set threshold during the exponential amplification phase.

Each biological replicate (n = 54) was run in triplicate on a 96-well plate. Two plates were required for each primer pair analyzed (B1, B2, AB1, AB2, ABC and YWHAG). Pooled non-implanted porcine endometrial RNA was diluted with nuclease-free H_2O (Ambion Inc., Austin, TX) to serial dilutions of known concentration (undiluted, 1:4 and 1:16) and triplicates of these dilutions were used as the internal control samples on each plate along with a "no template negative control" in which nuclease-free water is substituted for cDNA template.

Amplification efficiencies of each target fragment analyzed were calculated using the mean C_T values of the control dilutions: B1 (1.8), B2 (1.80), AB1 (2.4), AB2 (1.95), ABC (2.04), YWHAG (2.24). A linear plot of high (undiluted), mid (1:4 dilution) and low (1:16 dilution) average C_T (cycle threshold) values versus the logarithm of the respective dilution was generated and the slope of the line of best fit calculated. The means of sample triplicates and standard errors were calculated to determine outlying C_T values that were subsequently removed from the analysis. From these pruned averages,

fold changes were calculated relative to the abundance of the mid control (1:4 dilution) on the same plate using the formula:

$$(\text{Slope})^{\wedge(\text{mean mid control } C_T - \text{mean sample } C_T)}$$

to normalize the values on each plate, making inter-plate comparisons valid.

Primer pairs B2, AB2, and ABC3 used cDNA template derived from OSU isolated total, untreated RNA samples (n = 53) for qRT-PCR. Primer pairs B1 and AB1 used cDNA template derived from OSU isolated total, DNAse treated RNA samples (n = 53) for qRT-PCR.

Statistical Analysis

The data were analyzed as a completely randomized experimental design with two discrete independent variables: day and pregnancy status (pregnant or cyclic). The fold change relative to mid control was analyzed using the general linear model procedure (Proc GLM) of the Statistical Analysis System software (SAS Institute Inc,, Cary, N.C.). Day, pregnancy status and the interaction of day by pregnancy status were included in the model. The relative amount of expression (fold change calculated as described above) was analyzed as the dependent variable for each primer pair individually and used to estimate the effects of day (0, 5, 7.5, 10, 12, 13, 15, 17), status (cyclic or pregnant) and day by status interaction on the abundance of each target. Least squares means and pooled standard errors (SEM) were calculated using the LSMEANS procedure of SAS and individual treatment standard errors calculated as the LSMEANS

standard deviation divided by the square root of numbers of samples in the treatment ($s/\sqrt{n}$). The Levene test (HOVTEST) statement was used to test for homogeneity of variances under the means statement of SAS. Variances did not significantly differ for any of the primer sets analyzed (B1: P = 0.24; B2: P = 0.29; AB1: P = 0.12; AB2: P = 0.19; ABC: P = 0.35; YWHAG: P = 0.16).

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RESULTS

Northern Blot with DNA probe

Northern blotting with a DNA probe of pooled PGR PCR products revealed two distinct bands representing PGR-B and PGR-A. The PGR-C isoform was not detected in these samples by Northern blot (Figure 4.3). Interestingly, although the same quantity of total RNA was loaded into each well, the intensity of the bands differed between biological replicates. Additionally, although Northern blots are semi-quantitative, it appears that PGR-B and PGR-A are expressed at approximately the same abundance, as the bands are similar in intensity. The 28S band of rRNA migrated 4.4 cm from the well during electrophoresis and has a known size of 5.07 kb in pigs. The 18S band of rRNA migrated 5.7 cm from the well during electrophoresis and has a known size of 1.86 kb in pigs. Using these two points to create a slope for distance traveled in the gel to number of nucleotides, the larger transcript was measured at 4.5 kb, corresponding to the PGR-B isoform, and the smaller transcript was measured at 2.8 kb, corresponding to the PGR-A isoform (Figure 4.3). The possibility of cross-hybridization of DNA probes with

abundant 28S and 18S rRNA, however, could not be ruled out due to the similar sizes of these rRNA species with the expected PGR-B and PGR-A transcripts.

Northern Blot with riboprobe

Products in the RNA ladder of known size were separated by gel electrophoresis in tandem with the experimental samples. The distance each of these products migrated from the well was measured with a ruler. By plotting the size of the product against the distance traveled, the slope of the correlation between size and distance was calculated, making it possible to estimate the size of the PGR RNA products when the distance from the well was measured.

Northern blotting with a riboprobe of either B (PGR-B specific) or A (common to PGR-B and PGR-A) products revealed several bands (Figure 4.4). A high degree of non-specific binding, however, was detected using these riboprobes. In total RNA, both the B and A probes labeled the 28S and 18S rRNA transcripts so strongly that it obscured detection of any other transcripts (results not shown). In poly(A) RNA blots, however, removal of the 28S and 18S rRNA transcripts revealed the presence of a transcript of about 4.8 kb, which is approximately the correct size for the PGR-B isoform transcript. The second faint band seen in the poly(A) samples may be residual 18S rRNA but PGR-A transcript cannot be excluded. The PGR-C isoform was not detected in either total or poly(A) enriched RNA samples using either the B or A riboprobe. Additionally, Northern blots are semi-quantitative, and it appears that PGR-B and PGR-A are expressed at approximately the same abundance on d 0 and d 12, as the bands from the two biological replicates are similar in intensity (Figure 4.4).

Figure 4.3 Chemiluminescence of a Northern blot using total PGR DNA probe to detect transcripts in pig endometrium total RNA. Probe designed to common region of PGR-B and PGR-A transcripts. Lanes 1-8 = equal quantities of total endometrial RNA from 8 individual gilts. Arrows represent two major transcripts present; top = PGR-B located at 4.5 kb; bottom = PGR-A located at 2.8 kb. Both transcripts appear to be equally abundant within each sample. Variation in transcript abundance observed may be due to biological variation in expression between individual gilts or technical error.

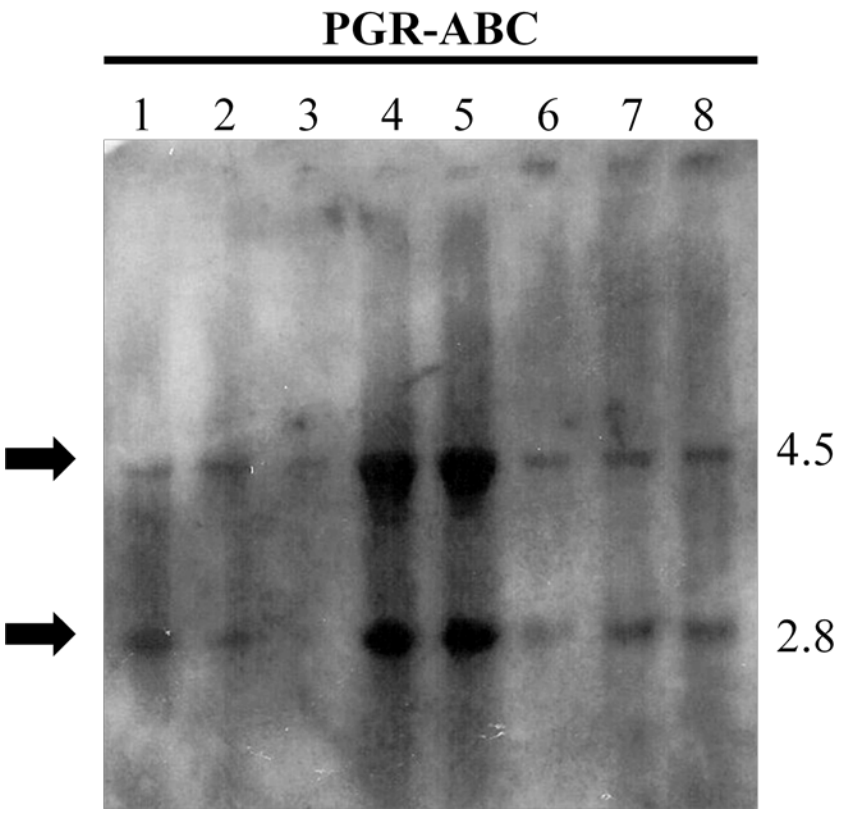
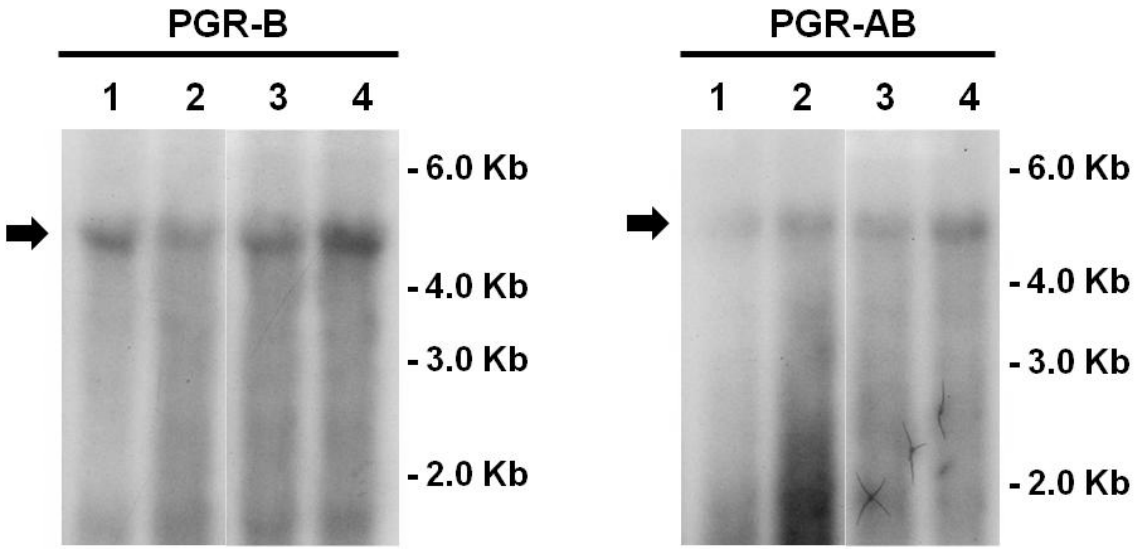


Figure 4.4 Autoradiographs of Northern blots using PGR-B or PGR-AB riboprobes to detect transcripts in poly(A) RNA of d 0 and d 12 cyclic pig endometrium. Probes designed to PGR-B specific region of PGR transcript (left) or PGR-B and PGR-A common region of PGR transcript (right). Lanes 1-2 = d 0 (estrus); lanes 3-4 = d 12. One major transcript detected by both probes was estimated to be 4.8 kb in length, suggesting the presence of only 1 PGR transcript likely corresponding to PGR-B. Transcript abundance does not appear to vary with day of estrous cycle.



RPA

The number of nucleotides in each band of the positive control sample was known. The distance each of these products migrated from the well during electrophoresis was measured with a ruler. By plotting the size against the distance traveled, the slope of the correlation between size and distance was calculated, making it possible to estimate the number of nucleotides of the protected fragment. This size could then be used to determine the location of the transcription initiation site.

Endometrium RNA was used for RPA analysis of transcription start sites from d 0 and d 12 (n = 3 per day). We were able to validate the presence of PGR-B, PGR-A, and PGR-C isoform transcripts in the endometrium using probes designed to span the transcription initiation sites. The B probe protected 2 major mRNA variants, one corresponding to the full-length PGR-B isoform mapping to the + 1 location of human PGR-B (*PGR-B.1*) and one alternative, downstream transcription initiation site for PGR-B (*PGR-B.2*; Figure 4.5). There are several stutter bands representing several proximal transcription initiation residues for the full-length PGR-B transcript (Figure 4.5), initiating at several sites between bases 291 and 314 (GQ903679.1). The alternative transcription initiation site for the B isoform (*PGR-B.2*) is located at base 371 (GQ903679.1). The *PGR-B.1* initiation site is located approximately 700 bases upstream of the translation start codon for the PGR-B protein isoform (Figure 4.9). The *PGR-B.2* initiation site is located approximately 650 bp upstream from the PGR-B start codon. Both *PGR-B.1* and *PGR-B.2* transcript variants were present in all samples and neither start site appeared to be dominant over the other between tissues harvested at d 0 or d 12

of the estrous cycle. Nor were any differences detected between total PGR-B transcript expression between d 0 and d 12 samples (Figure 4.4).

The original A riboprobe protected 3 mRNA fragments (Figure 4.6). The largest band represented the full-length protected PGR-B transcript hybridized to the complete riboprobe. Another band was observed at the expected *PGR-A* transcript initiation site (120 bases protected). The remaining band appeared to represent the same PGR-A transcript hybridized to a shorter, aberrantly transcribed riboprobe. Incomplete synthesis of the full-length riboprobe likely explains the result. The A riboprobe, therefore, was purified to eliminate any possible aberrant RNA molecules and RPA performed again. The results from the purified A probe demonstrate a dominant *PGR-A* transcription initiation site at 1046 bp (GQ903679.1; Figure 4.7) in addition to the full-length PGR-B transcript. The presence of some additional bands in one biological sample (Lane 4) may represent additional transcriptional initiation sites for the PGR-A isoforms. Additional samples are required to confirm additional bands. The position of the consistent (120 bp) PGR-A isoform transcription initiation is located approximately 470 bases upstream of the translation start codon for the PGR-A protein isoform (Figure 4.9). Using either the original or purified A probe, the PGR-A transcript represented by the 120 bp band did not appear to be expressed differently between d 0 and d 12 of the estrous cycle (Figures 4.6, 4.7).

The C riboprobe protected 3 mRNA variants (Figure 4.8). The full length *PGR-C.1* transcript is initiated at 2559 bp (GQ903679.1). Another transcript variant, *PGR-C.2*, is initiated at 2631 bp. Both sites are located in exon 1. The presence and size of these protected fragments indicates that normal splicing has occurred in these transcripts.

Figure 4.5 Autoradiograph of porcine PGR in poly(A) mRNA of endometrial tissue on d 0 and d 12 of the estrous cycle (n = 3) as detected by RPA with the B probe spanning PGR-B isoform transcription initiation site. A ribonucleotide ladder with known sizes is indicated in the lane labeled marker. Lane labeled PGR-B probe contains only radiolabeled probe to demonstrate full length probe size. The dash (-) indicates the negative control lane with unlabeled probe. Endometrium samples are in lanes 1-6: lanes 1-3 = d 0 (estrus); lanes 4-6 = d 12. Bracket indicates transcription initiation site for full-length PGR-B transcript (*PGR-B.1*). The smaller *PGR-B.2* band indicates an alternative downstream transcription initiation site for PGR-B isoform. Transcripts *PGR-B.1* and *PGR-B.2* appear similar in abundance and do not appear to vary with day of estrous cycle.

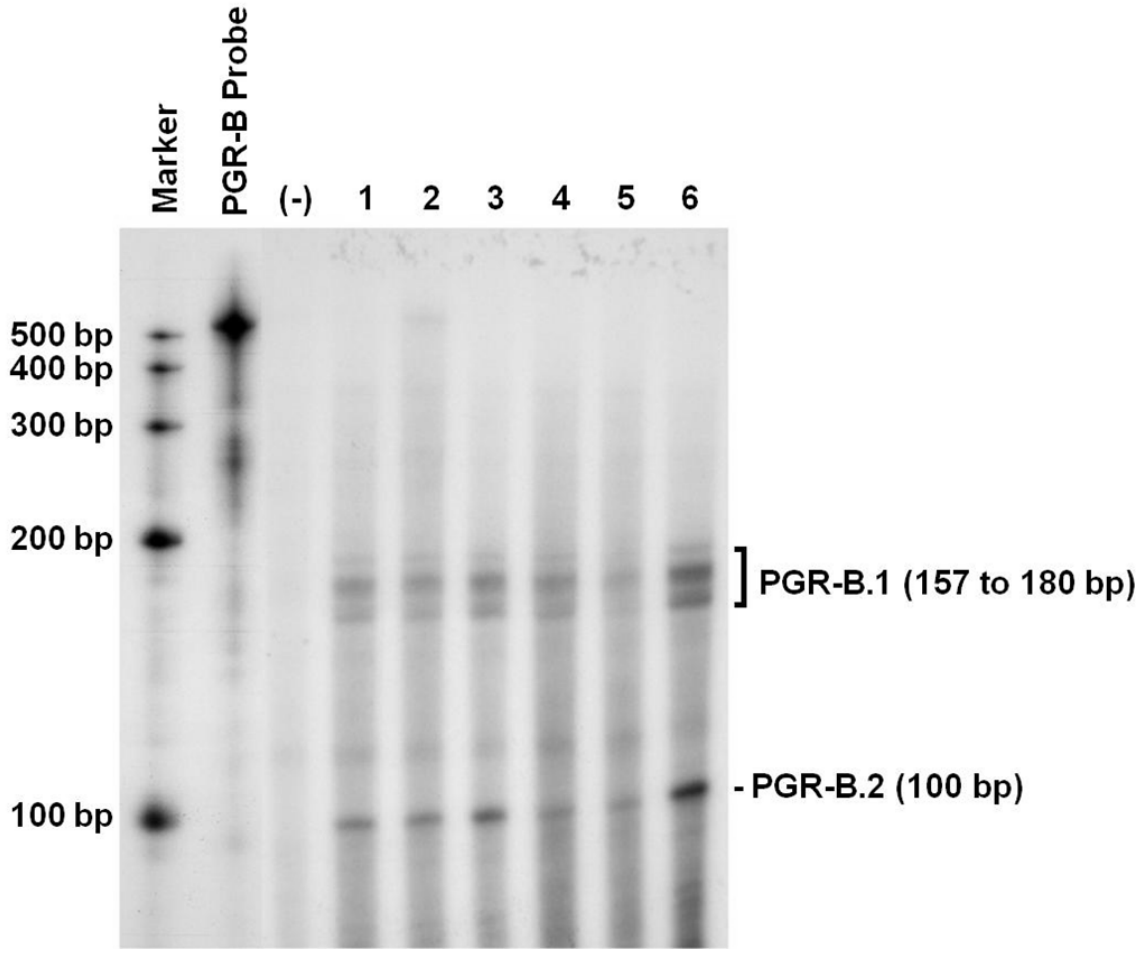


Figure 4.6 Autoradiograph of porcine PGR in poly(A) RNA of endometrial tissue on d 0 and d 12 of estrous cycle (n = 2, 3) as detected by RPA with the A probe spanning PGR-A isoform transcription initiation site. A ribonucleotide ladder with known sizes is indicated in lane labeled marker. Lane labeled PGR-A probe contains only radiolabeled probe to demonstrate full length probe size. The dash (-) indicates the negative control lane with unlabeled probe. Endometrium samples are in lanes 1-6: lanes 1-2 = d 0 (estrus); lanes 3-5 = d 12. The full-length PGR-B isoform initiation site is present at 240 bp with a PGR-A band at 120 bp. The protected fragment present at 102 bp is likely PGR-A transcript bound to a shorter, aberrantly transcribed PGR-A riboprobe seen at 180 bp in the PGR-A probe lane. Transcript abundance does not appear to vary with day of estrous cycle.

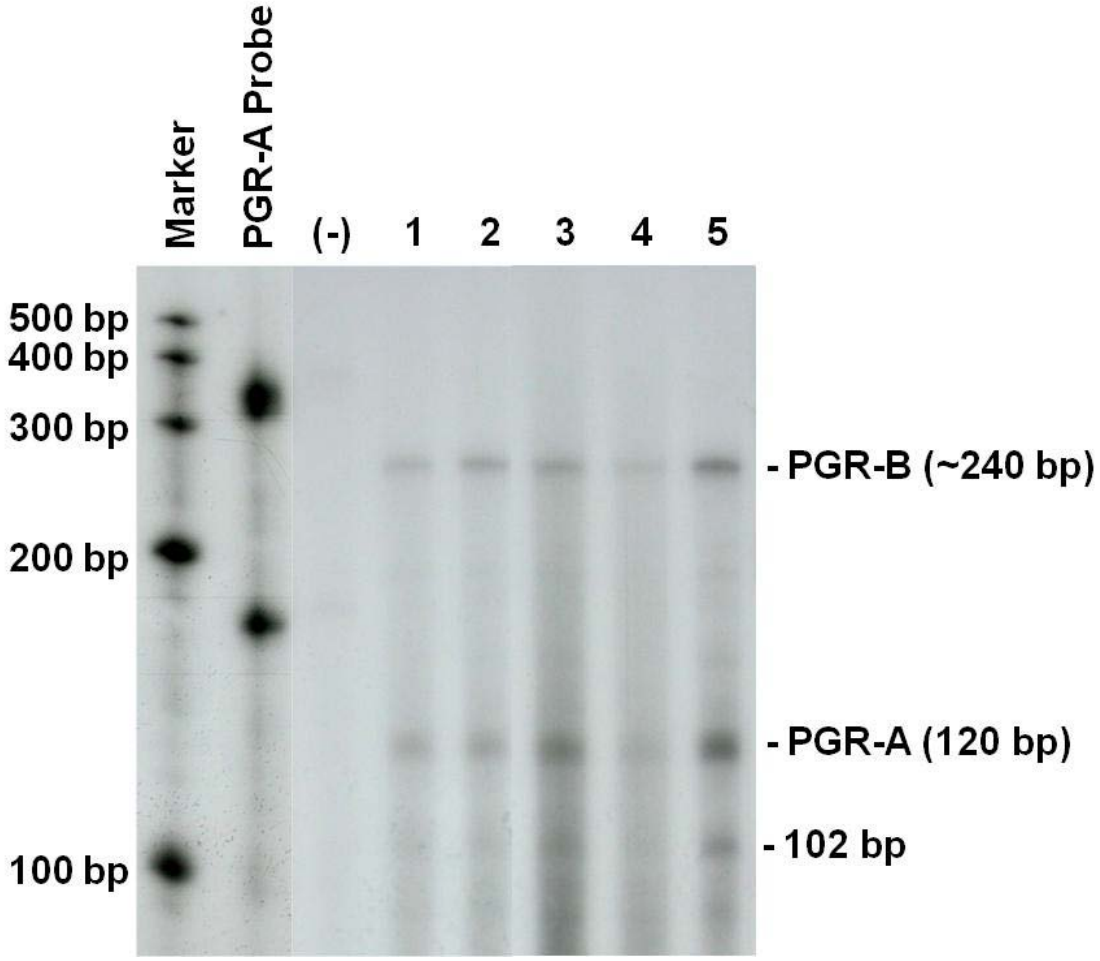


Figure 4.7 Autoradiograph of porcine PGR in poly(A) RNA of endometrial tissue on d 0 and d 12 of the estrous cycle (n = 1, 3) as detected by RPA with purified A probe spanning PGR-A isoform transcription initiation site. A ribonucleotide ladder with known sizes is indicated in lane labeled marker. Lane labeled PGR-A probe contains only radiolabeled probe to demonstrate full length probe size. The dash (-) indicates the negative control lane with unlabeled probe. Endometrium samples are in lanes 1-4: 1 = d 0 (estrus); 2-4 = d 12. A full-length PGR-B transcript is present at 240 bp and a single PGR-A transcript is present, with a 120 bp protected fragment in all samples tested and does not appear to vary with day of the estrous cycle. Additional products appear to be present in lane 4, but additional biological samples are required to determine if they are real transcription initiation sites.

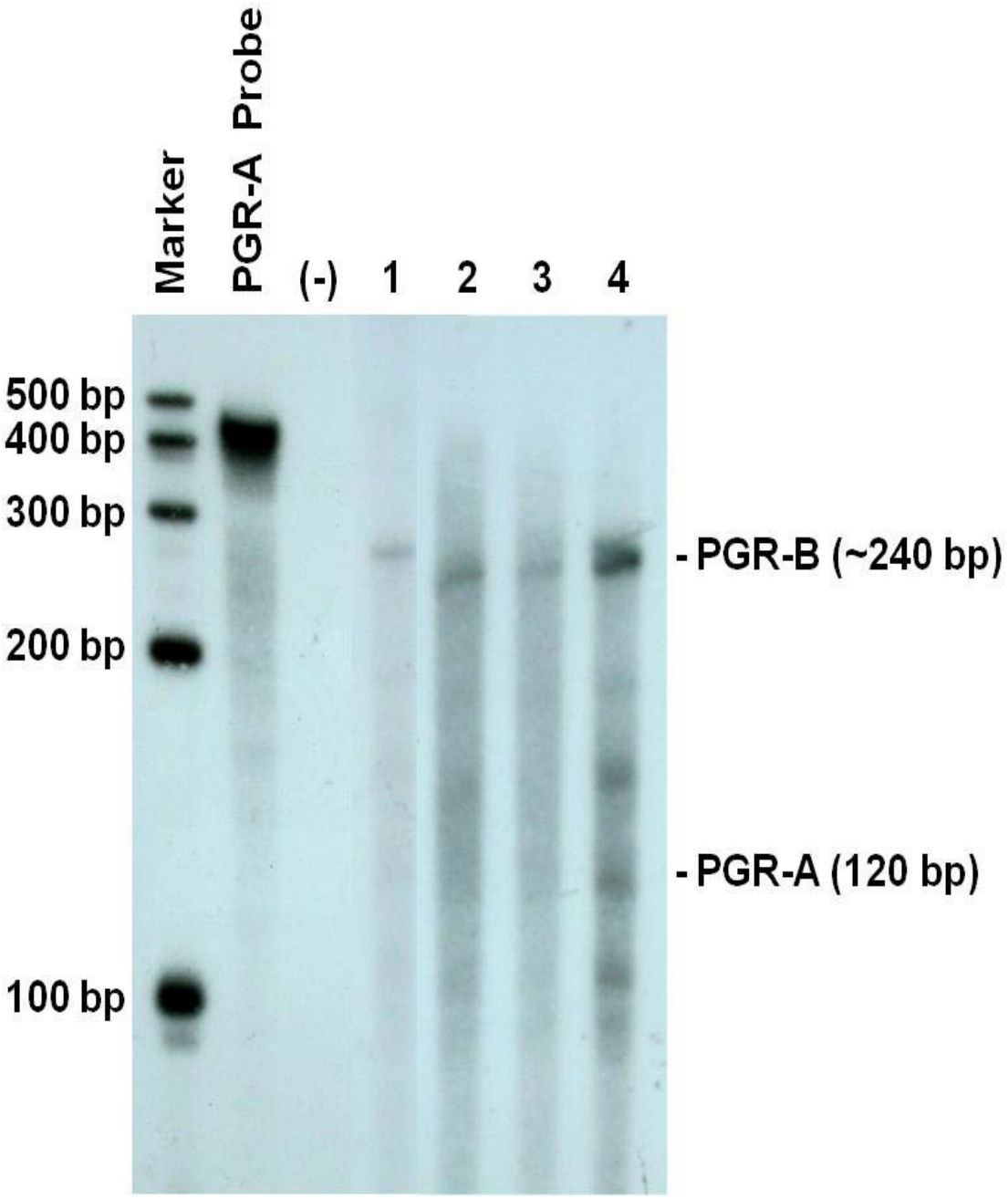
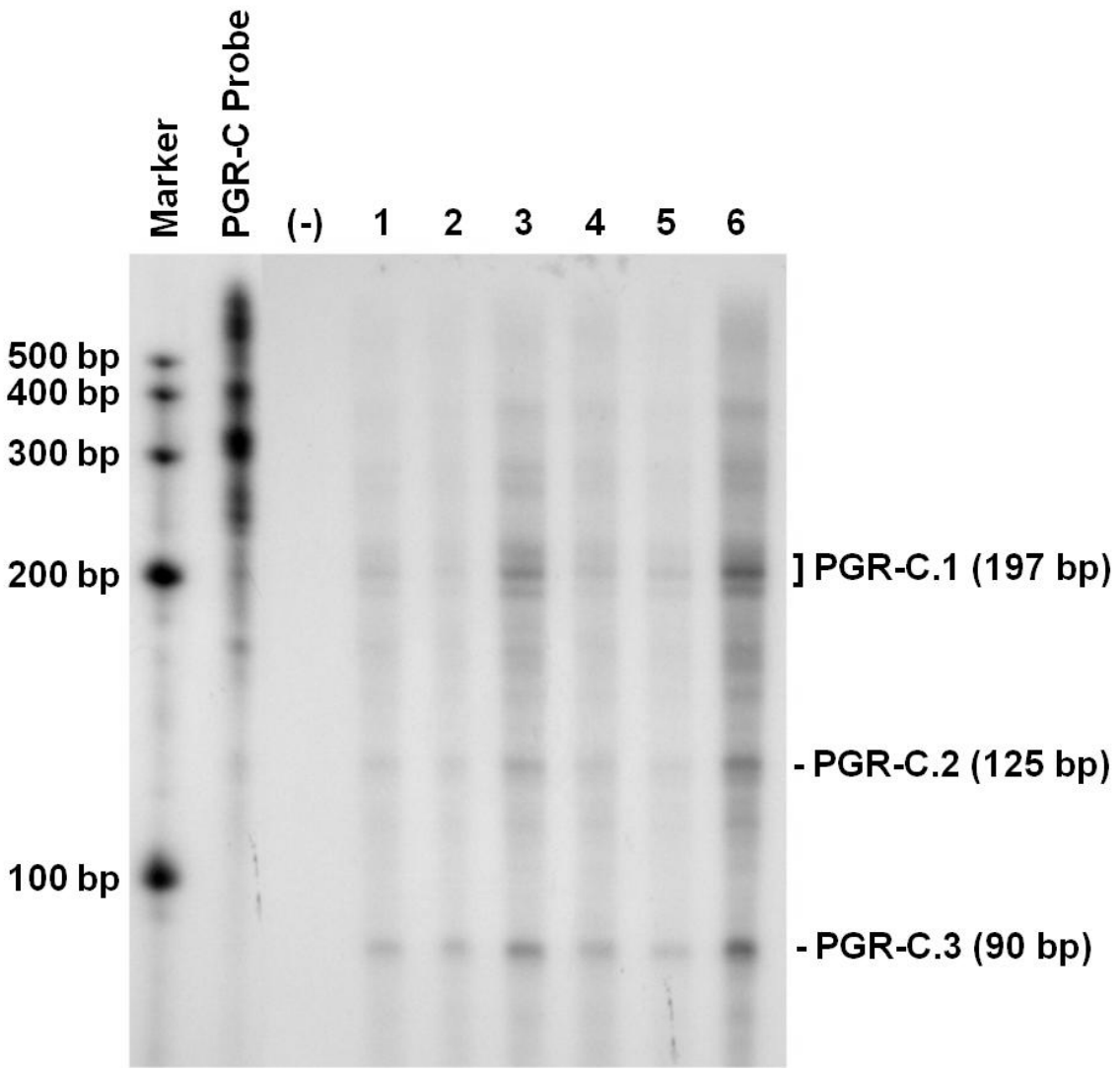

Marker
PGR-A Probe
(-)
1
2
3
4
500 bp
400 bp
300 bp
200 bp
100 bp
- PGR-B (~240 bp)
- PGR-A (120 bp)

Figure 4.8 Autoradiograph of porcine PGR in poly(A) RNA of endometrial tissue on d 0 and d 12 of the estrous cycle (n = 3) as detected by RPA with C probe spanning PGR-C isoform transcription initiation site. A ribonucleotide ladder with known sizes is indicated in lane labeled marker. Lane labeled PGR-A probe contains only radiolabeled probe to demonstrate full length probe size. The dash (-) indicates the negative control lane with unlabeled probe. Endometrium samples are in lanes 1-6: lanes 1-3 = d 0 (estrus); lanes 4-6 = d 12. Bracket represents multiple transcription initiation sites in exon 1 for *PGR-C.1*. The *PGR-C.2* transcript is an alternative initiation site located in exon 1. The *PGR-C.3* transcript is likely an artifact caused by unspliced transcript and not a real third initiation site for the PGR-C isoform. Abundance of *PGR-C.1* and *PGR-C.2* appears similar within samples and does not appear to vary with day of the estrous cycle.


Marker
PGR-C Probe
(-)
1
2
3
4
5
6
500 bp
400 bp
300 bp
200 bp
100 bp
] PGR-C.1 (197 bp)
- PGR-C.2 (125 bp)
- PGR-C.3 (90 bp)

There is, however, a protected product (*PGR-C.3*) that maps to base 2664, which corresponds to the splice site between exons 1 and 2. This is likely an artifact of transcripts that have not yet undergone splicing but we cannot be assured that it does not represent a real alternative transcript (Figure 4.8). The *PGR-C.1* and *PGR-C.2* transcript initiation sites are located, respectively, approximately 250 and 180 bases upstream of the translation start codon for the PGR-C protein isoform (Figure 4.9). The expression of PGR-C.1 and PGR-C.2 appeared to be approximately equal in samples and did not appear to vary in intensity between d 0 and d 12.

Figure 4.9 Location of transcription initiation sites and translation start codons for PGR-B, PGR-A and PGR-C isoforms as determined by RPA. The nucleotides of the 5 transcription initiation sites are represented in bold text with the name of the isoform and start site variant labeled in underlined text above (PGR-B.1, PGR-B.2, PGR-A.1, PGR-C.1 and PGR-C.2). The ATG translation start codons are represented in bold and underlined text with the isoform denoted in italics above (PGR-B, PGR-A and PGR-C).

```
  1 ttcttcttcc agggaaaaat ctttacaaaa ttgaaatctt gaaaattcag cttctgatag
 61 aatccattcg cttttccagt tagactgggc ataattctta attggtagta cacaatctaa
121 tatgcttacg aagttccatc ctcacagtac ctgtttttta gactagcggt tgggaatact
181 aactccagaa tttcagatct ggtccataat tggggcaggg aggggctttg ggcggagcct

                                                                _PGR-B.1_
241 ccggataggg aggaggcatt gttagaaagc tgtggggcta gtctactggc tgtcactact

    ____PGR-B.1  __
301 ctagttaagc ctttgtattt gaatgtgcga ggagggtctg ctgtgttagc agcattttca

            PGR-B.2
361 gtgagaacta gtctcacttg gcatttgagt ggaatctgca cctacaggcg tttcgtcccc
421 tccggactgc taagaaatgt gagggctgcg gagatgactg tcgccagcta tagggaaccg
481 ggaggtataa gactgttcag ggaatgggct gtgccgagag gccaaaatag ccccagggtt
541 ttagggagac gggaaatgta acacaggcag ggactggcag caacagggca ttgaagccgg
601 agaaaaagtc gggagataga gccgccagta attgctttcg aagccgccgc cactcgagtg
661 ctgacttgtg agtactctgc gtctcctgtc ttcagactga agtcggggaa ctctcttgga
721 gaactctccc agttaggaaa cgagatccct acaactacct tcgccggctc tccccacctg
781 cctcaaggac gtaacaacta tcacccatct cctgcctacc agacgctggg caagggcagg
841 agctgacagg tgccgccccc ttccgaccca ggaggtggag atttcacccc cctctccacc
901 gtcccgtcca gccaaattca acacctattt tctcctcccg ctgcccctat attcccgaca
961 ccccctcatc cttcccctat ccctcctccc cagagacagg ggaggagaaa aggggagtcc

            PGR-B                PGR-A.1
1021 aggtcgtcat gactgagctg aaggcaaagg atccccgggc tccccacgtg gcgggcagag
1081 cgccctcccc cacccagctc gggacgctgg gacgcccaga cacaggcccc tttcaggcga
1141 gccagacctc ggaagcgtcg cccgcagcct cggccatacc cctctccctg gacgggctac
1201 tcttccctgg gccctgccag ggacaggaac cagacgggaa gacgcaggac cagcagtcgc
1261 tgtcagacgt ggagggggcg tatcccacag ttgaagctac agagggtgct ggaggtggca
1321 gctctagacc ctcggaaaaa gacaccgggc tgctggacag tgtcttggac acgctactag
1381 cgccctcagg tcccgggcag agccacgcca gccctcccgc ctgcgaagcc accagccctt
1441 ggtgcttgtt tggctctgag cttccccagg acgctcgggt tgcccttcc acccagggag

                    PGR-A
1501 tattgcccct gctcatgagc cggccagagg gcaaggctgg cgacagctcc gggacggcag
1561 ccgcccataa agtgctatcc aggggtctgg caccgtcccg gcagctgctg ccctcgactg
1621 ccgggagcca tcactggccg gcggccgcag tgaagccctc tccgcagccc gccgtggtgg
1681 aggtggagga ggaggatgac tccgagtccg agggctccat ggttccactt ctgaagggta
1741 aaccccggcc tgcaggaggc acggctgccg gaacaggaac cccgaccggt gctcctggga
1801 cggccgcagg aggcgtcgcc ctggtcccca aggaagatgc ccgcttctcg gcgcccaggg
1861 gcgccctggc ggagcacgat gcgtcagggg cgcccgggcg ctcccctctg gccactacgg
1921 tgatggattt tatccacgtg cccatcctgc cgctcaacac ggcctttctg gccgcccgca
1981 cccggcaact gctagagggg gataactatg acggcggggc cccggctgtc agcgcctttg
2041 caccgccgcg gggctcgccc tcggccccgt ccgcctccgt caccgccggc gacttccccg
2101 actgcgcgta ctctgcagac gacgagccca aggacgacgc attcccgctc tatggcgact
2161 tccagccgcc cgccctgaag atcaaggaag aggaggaagg cgccgaggcc gccgcgcgct
2221 ctccgcggtc gtacctggtg gcgggtgcca accctgccgt cttcccggat tttccgctgg
2281 cgccgccgtc gctgcctccg cgagcgtcgt ccagacctgg ggaagcctcg gcggcggtcg
2341 cacccaccag tgcctcggta tcgtcggcgt cctcgtcggg gtcggccttg gagtgcatgt
2401 tgtataaggc ggagggcgcg ccgccccaac agggcccgtt cccgcctccg ccctgcaagc
2461 cgccgagcgc cggcgcctgc ctgctgccgc gggacagcct gccgtccacc tcggcctccg

                                                    PGR-C.1
2521 ccgccgccgc cgccgccgcc gccgccgggg cggctcctgc gctctaccag ccgcttggcc
```

PGR-C.2

2581 ttaacgggct cccgcagctc ggctaccagg ccgcggtact caaggagggc **c**tgccgcagg
2641 tgtaccagcc ctatctcaac tacctgaggc cggattcaga agccagccag agcccacagt
2701 acagcttcga gtcattacct cagaagattt gtttaatctg tggggatgaa gcatcaggct

PGR-C

2761 gtcattatgg tgtccttacc tgtgggagct gtaaggtctt ctttaaaagg gca**atg**gaag

Real-time PCR with B1 and AB1 primer sets

There was a tendency for an effect of day on PGR-B expression ($P < 0.10$), due to increased presence of PGR-B mRNA on d 0 (0.52 ± 0.07) and d 5 (0.51 ± 0.07) compared with d 7.5 (0.31 ± 0.07) and d 15 (0.30 ± 0.07). PGR-B transcript abundance was intermediate on d 10, 12, 13 and 17 (Figure 4.10). There was a day by status interaction effect on PGR-AB mRNA. The PGR-AB mRNA remained low through d 13 (0.13 ± 0.01; d 0 to 13; cyclic and pregnant) and increased on d 15 in both pregnant (0.90 ± 0.07) and cyclic (0.41 ± 0.07) pigs. The PGR-AB mRNA remained elevated in pregnant pigs on d 17 (0.33 ± 0.07; Figure 4.11).

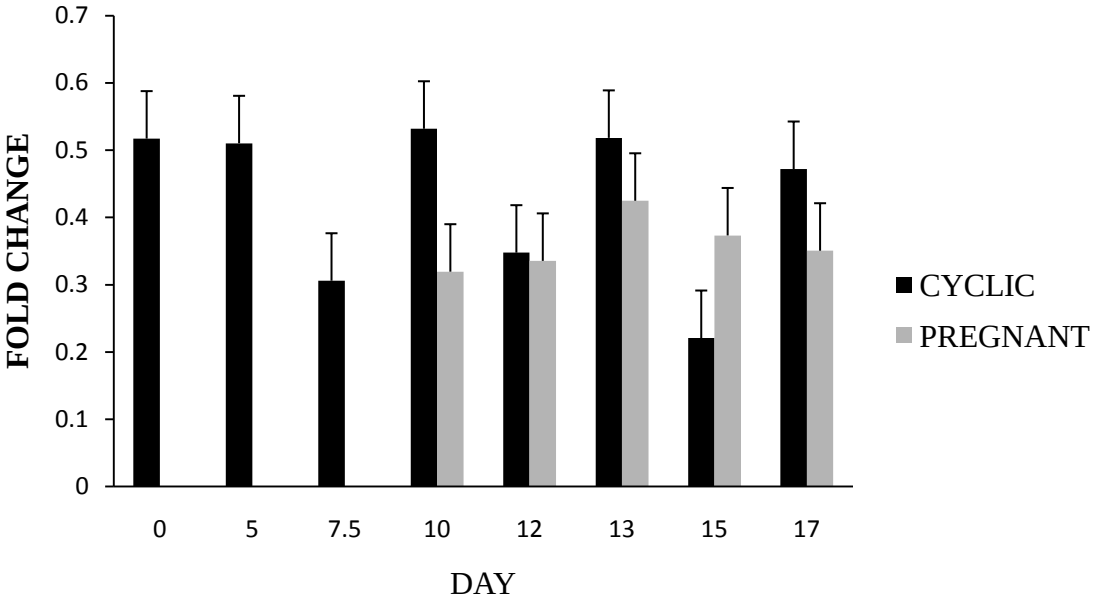
Real-time PCR with YWHAG primer set

There was no significant effect of day, status or day by status interaction on YWHAG expression ($P = 0.72$) in the pig endometrium (Figure 4.12). Expression of YWHAG appears to be constitutive from d 0 – d 17 in both cyclic and pregnant pigs.

Real-time PCR with B2, AB2 and ABC primer sets

There was an effect of day by status interaction on PGR-B expression ($P < 0.001$), due to increased presence of PGR-B mRNA on d 0 (4.73 ± .51) and d 5 (3.73 ± .51) compared with d 7.5 (2.58 ± .51) and d 15 (2.46 ± .51) in cyclic animals. The remaining days were intermediate. In pregnant pigs abundance of PGR-B mRNA in pregnant pigs was low on d 10 – d 13 with a significant increase on d 15 (5.16 ± 0.51) and then returns to low abundance on d 17 ($P < 0.001$; Figure 4.13). There was an effect of day on PGR-AB expression ($P = 0.04$), due to increased abundance of PGR-AB mRNA on d 0 (4.23 ±

Figure 4.10 Least squares means for fold change in mRNA abundance of PGR-B in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 53) of the estrous cycle using B1 primer pair. Shown top: error bars represent pooled SEM (0.07), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. Abundance of PGR-B is increased on d 0 and d 5 compared with d 7.5 and d 15 ($P < 0.10$), with d 10, 12, 13 and 17 intermediate.


FOLD CHANGE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

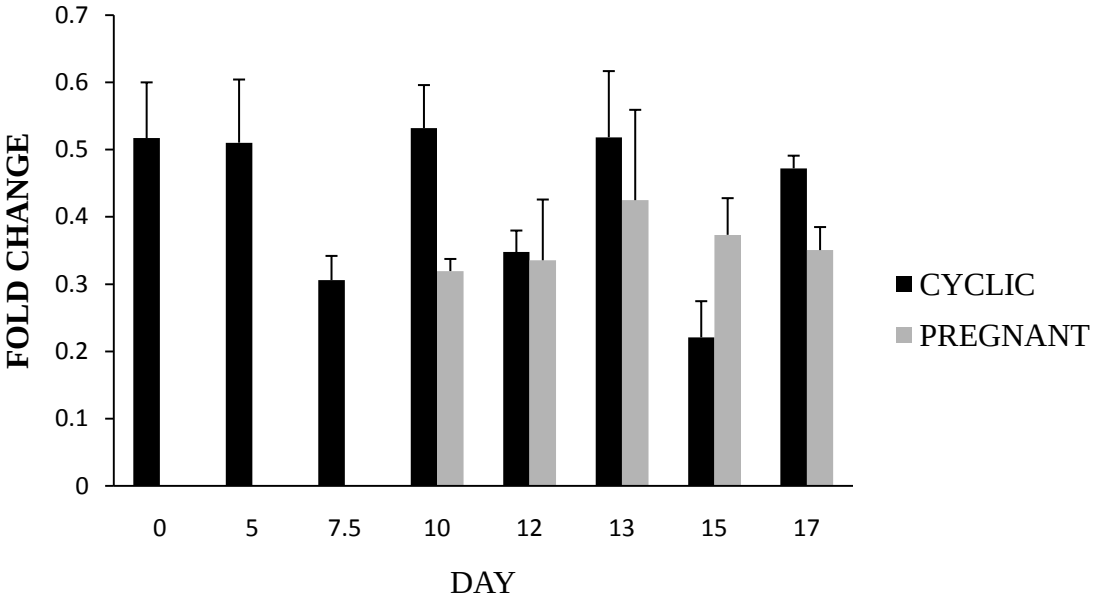
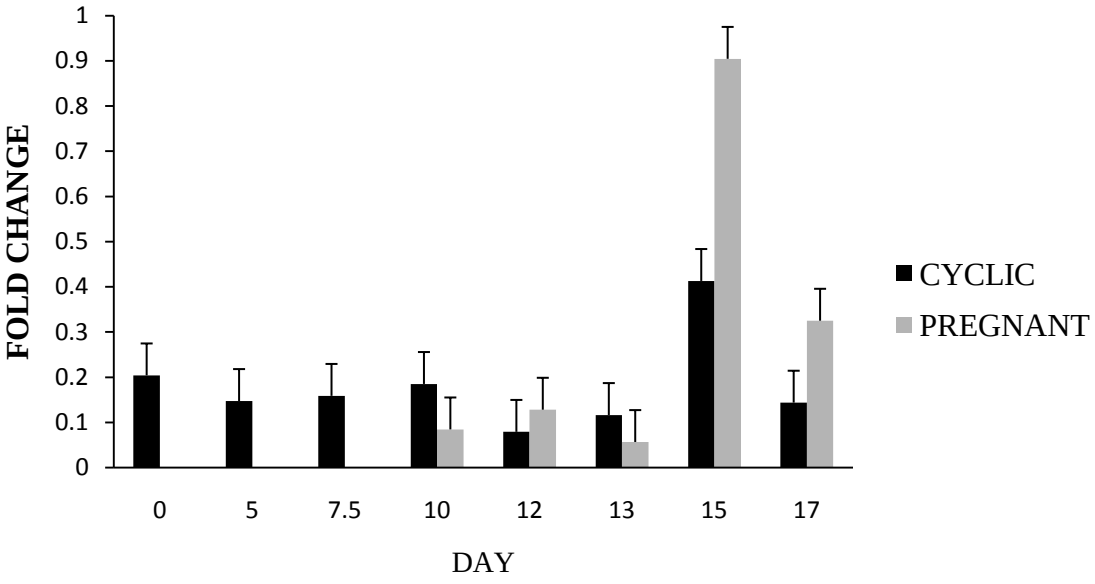

FOLD CHANGE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

Figure 4.11 Least squares means for fold change in mRNA abundance of PGR-AB in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 53) using AB1 primer pair to amplify common sequence of PGR-B and PGR-A. Shown top: error bars represent pooled SEM (0.07), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. Abundance of PGR-AB is increased on d 15 in both pregnant and cyclic pigs over all d 0 – 13 samples ($P < 0.001$) and remained elevated in pregnant pigs on d 17 ($P < 0.001$), while their cyclic counterparts returned to baseline levels of expression.


FOLD CHANGE
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

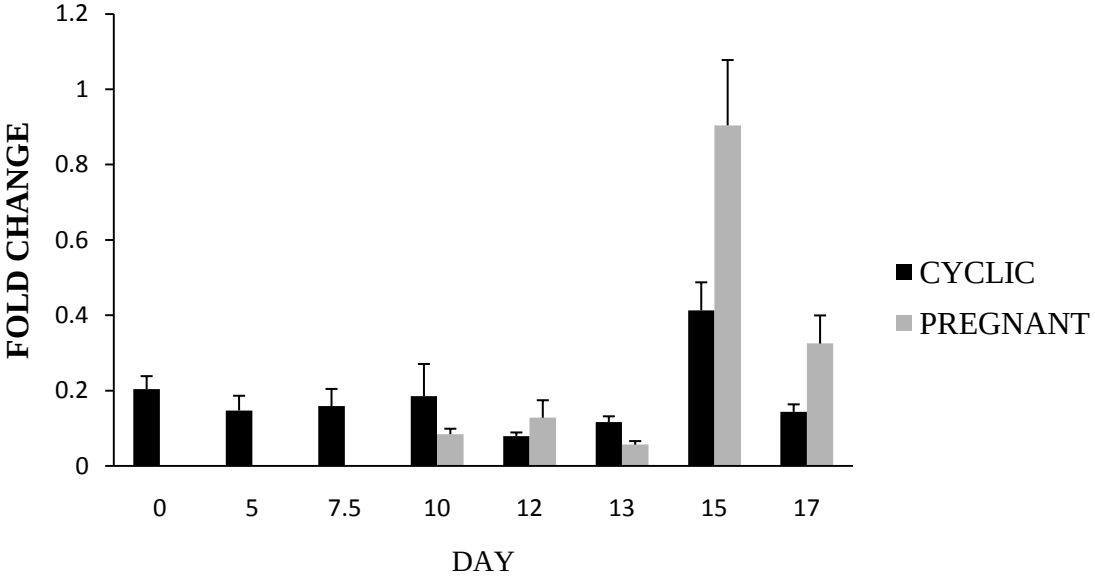
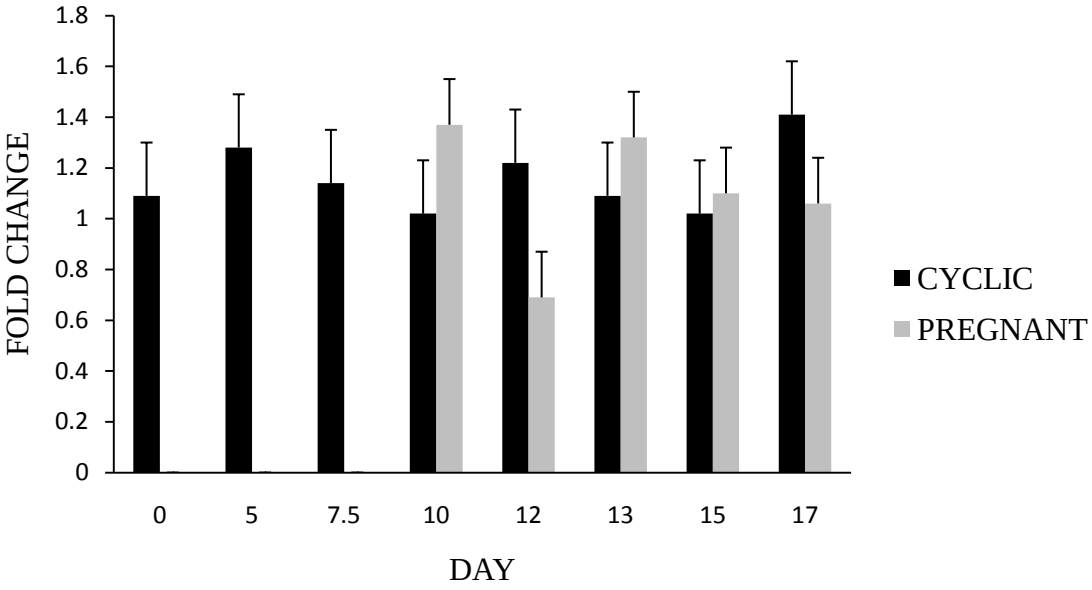

FOLD CHANGE
1.2
1
0.8
0.6
0.4
0.2
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

Figure 4.12 Least squares means for fold change in mRNA abundance of control gene YWHAG in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 54) using YWHAG primer pair. Shown top: error bars represent pooled SEM (cyclic = 0.21, pregnant = 0.18), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. There was no significant effect of day, status or day by status interaction on YWHAG expression (P = 0.72).


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
FOLD CHANGE
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

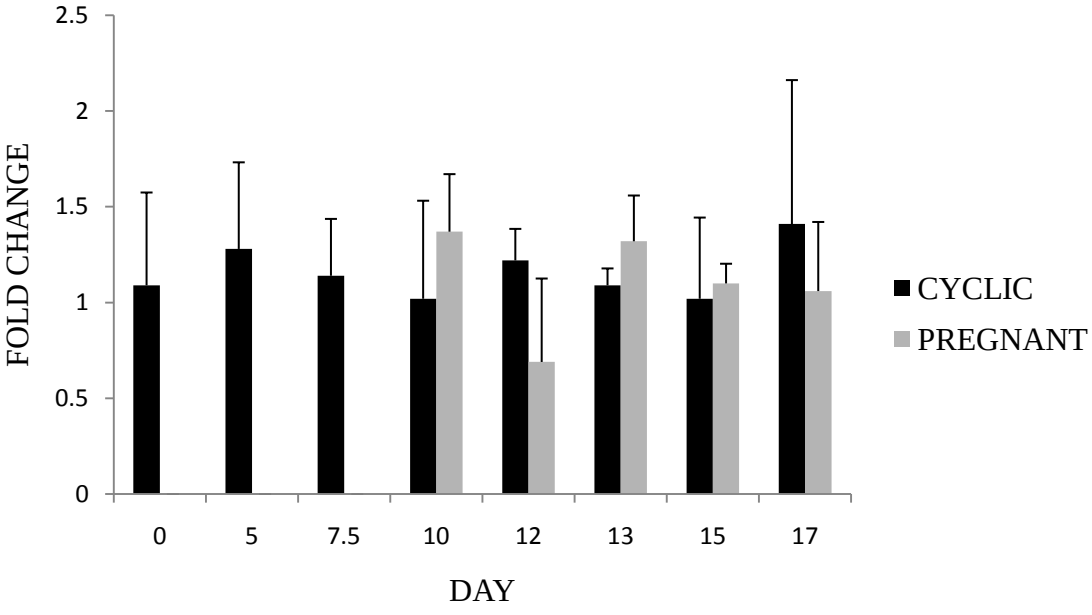
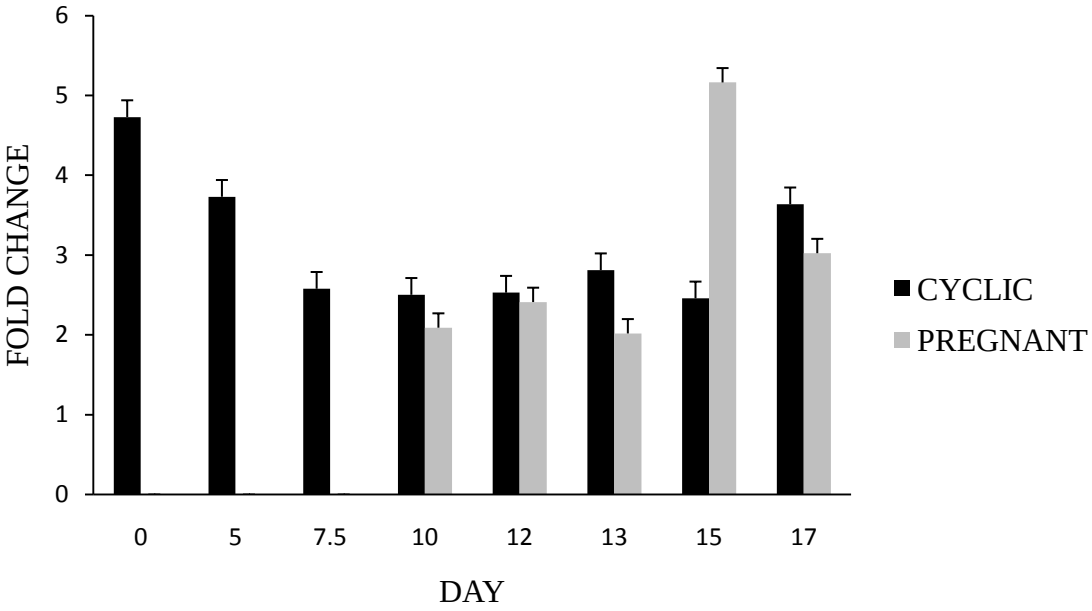
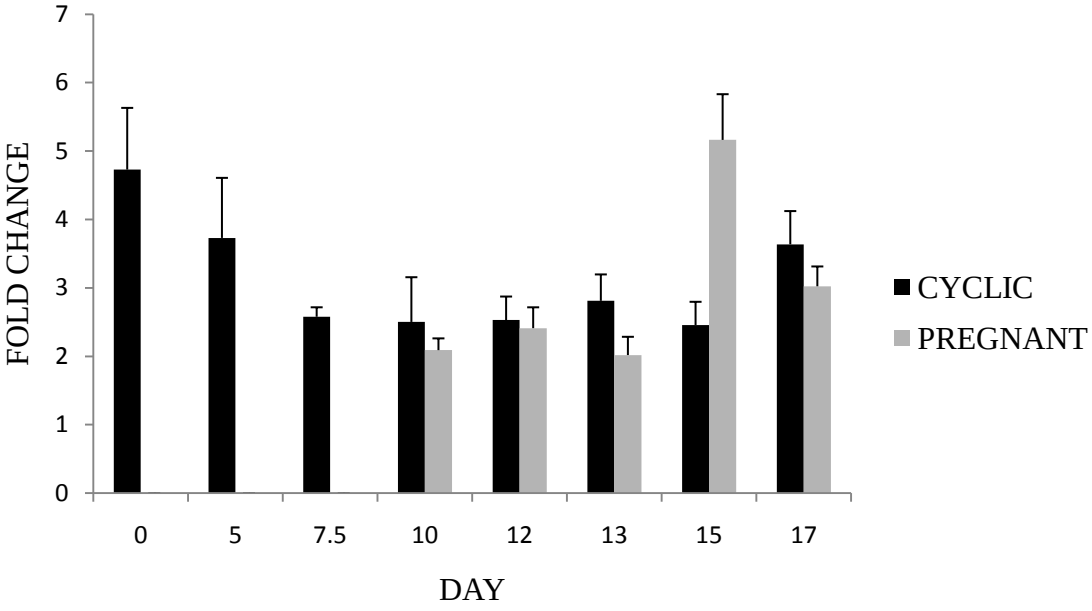

2.5
2
1.5
1
0.5
0
FOLD CHANGE
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

Figure 4.13 Least squares means for fold change in mRNA abundance of PGR-AB in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 54) using the B2 primer pair to amplify common sequence of PGR-B and PGR-A isoforms. Shown top: error bars represent pooled SEM (0.51), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. Abundance of PGR-B mRNA in cyclic pigs decreases from d 0 to d 7.5 where abundance plateaus until d 15 and then increases again on d 17. Abundance of PGR-B mRNA in pregnant pigs was low on d 10 – d 13 with a significant increase on d 15 and then returns to low abundance on d 17 ($P < 0.001$).


FOLD CHANGE
0
1
2
3
4
5
6
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

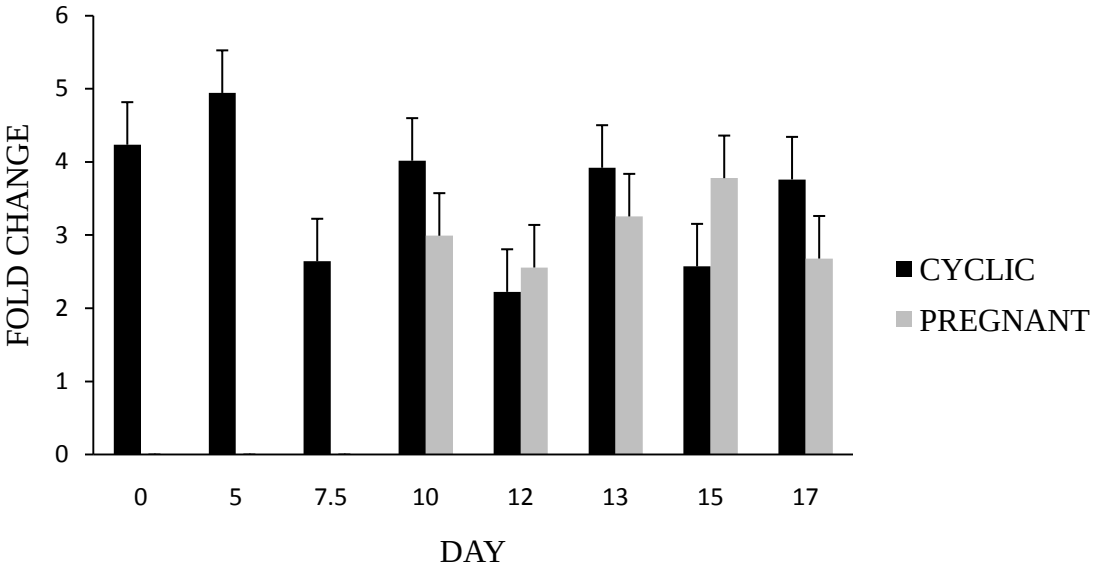

FOLD CHANGE
0
1
2
3
4
5
6
7
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

0.58) and d 5 (4.94 ± 0.58) compared with d 7.5 (2.64 ± 0.58), d 12 (2.22 ± 0.58) and d 15 (2.57 ± 0.58). The remaining days were intermediate in cyclic animals. Abundance in pregnant animals remained constant on d 10 – 17. (Figure 4.14). There was no significant effect of day, status or day by status interaction on PGR-ABC expression (P = 0.92), due to high variation between biological replicates resulting in large standard error values (Figure 4.15).

DISCUSSION

Several PGR mRNA variants have been detected by Northern blot in various species. In particular, a large mRNA variant of greater than 10 kb in length is detected only in some species. The size of these variants ranges from 11.1 kb to 14 kb (Kraus and Katzenellenbogen, 1993; Turgeon et al., 1999; Wei et al., 1988; Carroll et al., 1993; O'Brien et al., 1998). Some of this variation may be due to inexact measuring of band migration on the Northern blot or uneven running of the gel. This large mRNA transcript has been detected in both rats and humans in both total and polyadenylated RNA. In poly(A) RNA from both the rat pituitary and uterus, a DNA probe directed to the PGR-B and PGR-A hormone binding domain common region was able to detect a large species (> 10 kb; Kraus and Katzenellenbogen, 1993; Turgeon et al., 1999). In total RNA extracted from human adipose, uterus, meningiomas and T47D carcinoma cell line, when a DNA probe directed to the common region of PGR-B and PGR-A was used, a large RNA species > 10 kb was observed (Wei et al., 1988; Carroll et al., 1993; O'Brien et al.,

Figure 4.14 Least squares means for fold change in mRNA abundance of PGR-ABC in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 54) using AB2 primer pair to amplify common sequence of PGR-B, PGR-A and PGR-C isoforms. Shown top: error bars represent pooled SEM (0.58), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. There was an effect of day on PGR-AB expression (P = 0.04), due to increased abundance of PGR-AB mRNA on d 0 and d 5 compared with d 7.5, d 12 and d 15. The remaining days were intermediate in cyclic animals. Abundance in pregnant animals remained constant on d 10 – 17.


FOLD CHANGE
6
5
4
3
2
1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

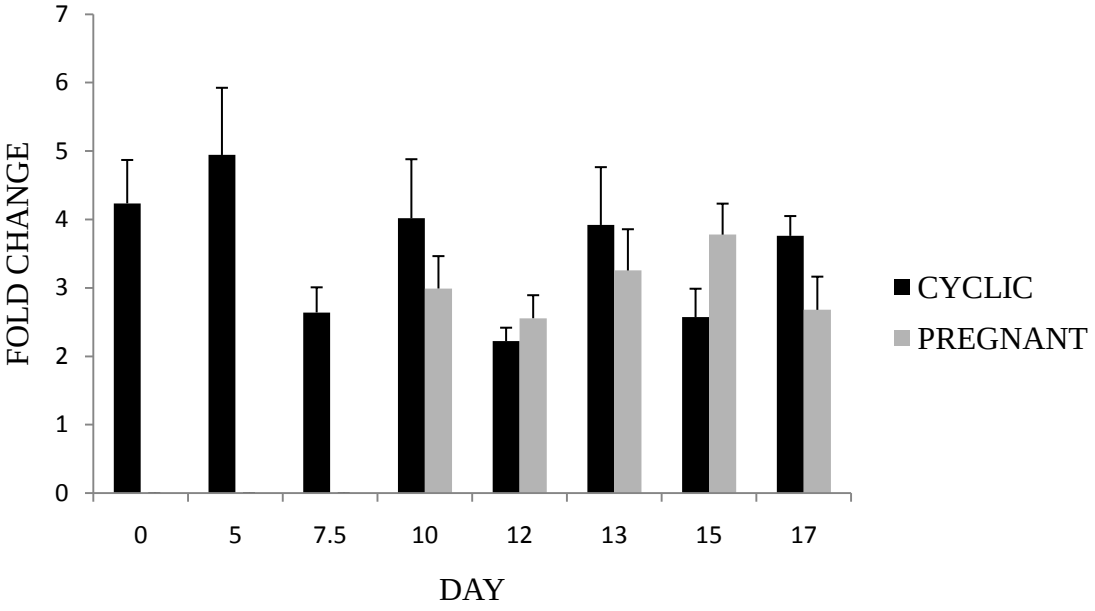
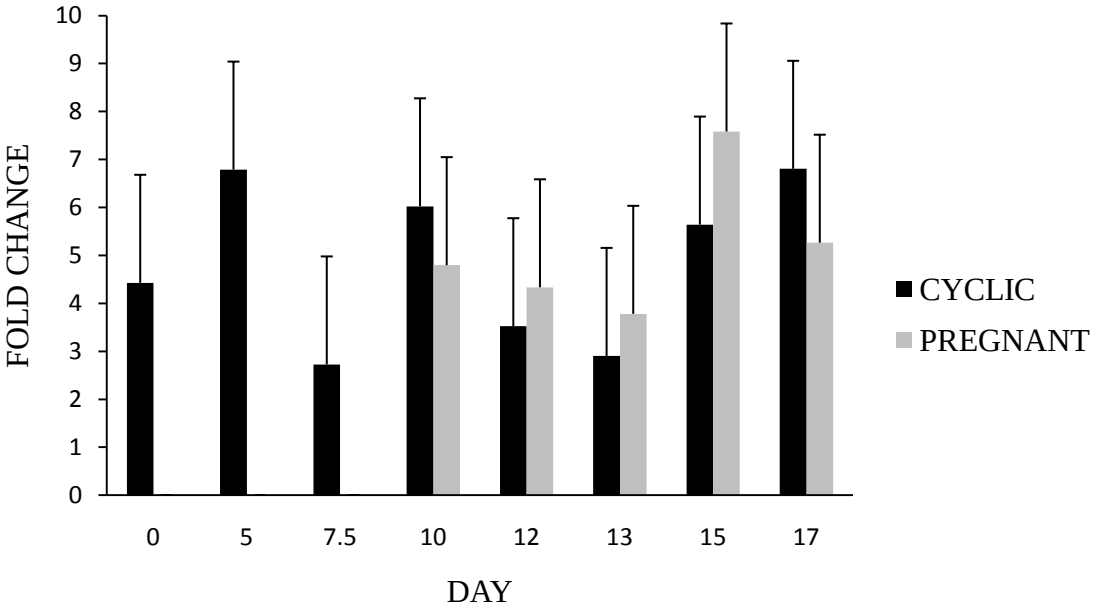
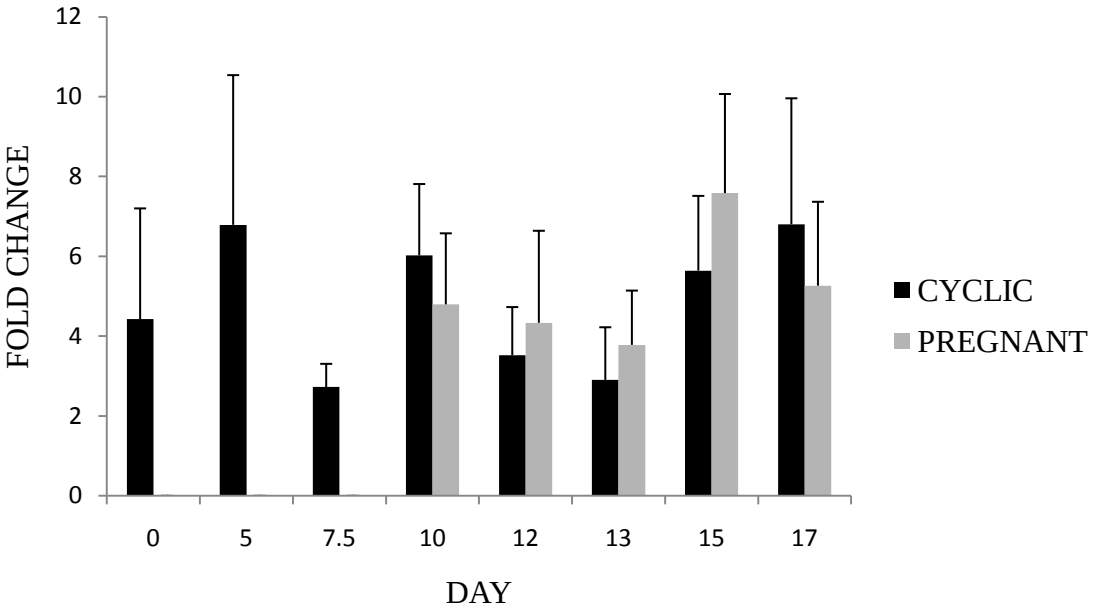

FOLD CHANGE
7
6
5
4
3
2
1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

Figure 4.15 Least squares means for fold change in mRNA abundance of total PGR in cyclic and pregnant endometrium at d 0, 5, 7.5, 10, 12, 13, 15 and 17 (n = 54) using ABC primer pair to amplify common sequence of PGR-B, PGR-A and PGR-C isoforms. Shown top: error bars represent pooled SEM (2.25), bottom: error bars represent individual treatment standard error. Day 0 denotes estrus. Fold change is calculated as the least squares mean Ct of sample triplicates relative to a control sample of pooled non-implanted porcine endometrial RNA diluted at a 1:4 ratio with nuclease-free H_2O. There was no significant effect of day, status or day by status interaction on PGR-ABC expression (P = 0.92).


FOLD CHANGE
10
9
8
7
6
5
4
3
2
1
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT


FOLD CHANGE
12
10
8
6
4
2
0
0
5
7.5
10
12
13
15
17
DAY
CYCLIC
PREGNANT

1998). In mice and bovine, however, this large mRNA transcript was not detected by Northern Blot. Using either total of poly(A) RNA, a DNA probe directed against the common PGR-B and PGR-A region of PGR was unable to detect any species of RNA over 7 kb in length (Tan et al., 1999; Kurita et al., 2001). Likewise, using total RNA, a DNA probe directed against the hormone binding domain of PGR which is common to both the PGR-B and PGR-A isoforms was unable to detect any species of RNA over 7 kb in length in bovine follicle wall, CL or myometrial tissue (Jo et al., 2002).

Because the large transcript of RNA (> 10 kb) was detected in both samples of total and poly(A) RNA, using a probe capable of recognizing PGR-B, PGR-A and even PGR-C isoforms, the cause for the presence of the large RNA variant in humans and rats but not in mice and cows is not immediately apparent. The observation may be an artifact of RNA isolation or denaturation in preparation for the Northern blot procedure. Another complication in investigating when the large species is present is the practice of cropping Northern blot pictures for publication and also excluding the ladder used to determine band size of the RNA transcripts detected. The presence, purpose, importance and isoform specificity of this large RNA transcript, therefore remains a question to be investigated. It does seem clear, however, that no large transcript was present in the Northern blot experiments presented here.

Results were not consistent between Northern blots conducted with DNA probes using total RNA and Northern blots conducted with riboprobes using poly(A) enriched RNA target. In theory use of a riboprobe and poly(A) enriched RNA should both increase technique sensitivity over DNA probe and total cellular RNA. However, it would appear that PGR-A was capable of being detected in the latter system rather than

the former. There are several possible explanations. First, the riboprobe was observed to hybridize very strongly to rRNA. It is likely that the DNA probe would share a similar affinity and that the lower molecular weight band detected by DNA probe is, in fact, 18S rRNA rather than the PGR-A isoform. Another possibility is that the PGR-A transcript is susceptible to degradation and the process of poly(A) isolation of total RNA destroyed the transcript of interest before it could be assessed. Finally, secondary structure of PGR RNA molecules had presented previous challenges. The DNA probe provides more comprehensive coverage of the PGR transcript. The riboprobes are targeted to specific regions. It is therefore possible that complex secondary structure of PGR-A RNA precludes hybridization of complementary sequence in the specific region targeted by the A riboprobe, while the increased coverage of PGR sequence by the DNA probe permits complementary hybridization in regions of RNA with less complex secondary structure.

Another observation of note in analysis of the Northern blot results was that the intensity of PGR bands did not seem to correlate to the level of expression detected by qRT-PCR. Samples for Northern blotting with a DNA probe were selected based on high abundance of PGR detected by qRT-PCR to be expressed at a higher intensity on the autoradiograph. Our Northern blots, however, were not correlated between the two assay techniques. This indicates that caution needs to be taken when verifying results by Northern or by qRT-PCR to ensure consistency among methods. The discrepancy may also indicate the sensitivity of specific PGR mRNA transcripts to degradation.

Transcription initiation sites were identified for PGR-B, PGR-A and PGR-C isoforms by RPA. The PGR-B transcription initiation site locations (*PGR-B.1* and *PGR-B.2*) were consistent with cDNA sequence construction, concluding that a PGR

transcription initiation site was location between bases 1 and 470 in Chapter 2. The observation of PGR-C transcription initiation sites indicate that there is a transcript variant initiated downstream of the PGR-A isoform translational start codon (PGR-C). This is consistent with the qRT-PCR data which implied the existence of a PGR-C isoform in addition to PGR-B and PGR-A when total PGR was analyzed.

The evidence of PGR-C demonstrated by the RPA is more direct, pinpointing a location at which transcription is initiated for a PGR-C isoform. The presence of a PGR-C isoform, however, is not indicated by the results of the Northern blots performed with either DNA- or riboprobes. One possible explanation is that PGR-C transcripts are unstable and easily degraded and does not withstand the duration of the electrophoresis to which it is subjected in Northern blots. The length of time during which the sample RNA migrates through a gel is much longer in the Northern blot protocol described above than in the RPA protocol. Likewise, the RNA is much less exposed to conditions favorable for degradation in qRT-PCR reactions in which the samples are prepared quickly and on ice for amplification. If, indeed, PGR-C is an unstable transcript, there is the possibility that it may not have much, if any, effect *in vivo*.

Different primer sets for the PGR-B and PGR-AB isoforms resulted in different patterns of mRNA expression using qRT-PCR. In an effort to be more precise in measuring changes in PGR expression during the estrous cycle and early pregnancy measures were taken to control contamination. Aliquots of total RNA samples were treated with DNAse and a second cDNA synthesis was performed (n = 53). Primer pairs B1 and AB1 were designed to ensure isoform specificity. There was an effect of day on the most 5' primer set (B1), specific to PGR-B. PGR-B was present at high

concentration on d 0 and d 5 and was decreased on d 7.5 in cyclic and d 15 in both cyclic and pregnant pigs. The remaining days had intermediate PGR-B abundance in both cyclic and pregnant pigs. The PGR-B isoform was expected to be down-regulated by the presence of high concentrations of P4 secreted by the CL after ovulation between d 0 and 7.5. The similar pattern of expression in both cyclic and pregnant endometrium was also in accordance with results of previous studies (Geisert et al., 1994; Ross et al., 2010; Sukjumlong et al., 2009). The level of PGR-B expression would be expected to recover after mid-cycle regression of the CL due to decreasing P4 concentration and increasing E2 concentration from the new cohort of developing follicles. This recovery was not observed by d 17 in this study. We would expect, however, to observe recovery of PGR-B abundance to d 0 concentrations between d 18 and 21 of the cycle, if the samples were available.

The PGR-AB isoform was detected using qRT-PCR by both the B2 and AB1 primer sets. The B2 primer set detects the 5' UTR of the PGR-A isoform and the AB1 primer set is located downstream in the common coding region of PGR-B and PGR-A. Accordingly, the results of the qRT-PCR analysis were similar in pregnant pigs for both primer sets. There was a day by status interaction effect on PGR-AB mRNA abundance in the endometrium. In pregnant pigs low, constitutive expression of PGR-AB was observed on d 10 – 13 and 17 with a significant increase in mRNA abundance on d 15 using both B2 and AB1 primer sets. The pattern of expression in cyclic pigs differed between B2 and AB1 primer sets. The AB1 primer pair detected a pattern of expression mirroring that of the pregnant pig, with low, constitutive expression of PGR-AB on d 0 – 13 and 17 and a significant increase in abundance on d 15. The increase of PGR-AB on d

15 measured by the AB1 primer pair was greater in pregnant pigs than in cyclic pigs. Using the B2 primer pair, however, abundance of PGR-AB was high in cyclic pigs on d 0, steadily decreased to d 7.5 and remained lowly abundant until recovery on d 17. This pattern of expression more closely resembles the pattern observed in the PGR-B isoform using the B1 primer set in both cyclic and pregnant animals.

Taken together, these results may suggest that during pregnancy, a different uterine environment and presence of different coactivator proteins in the uterus may cause the transcription of the PGR-A isoform to begin at a nucleotide further downstream (3') from the transcription initiation site for the PGR-A isoform utilized in cyclic pigs. The B2 primer pair may be located in the unique position to detect the longer 5' UTR of the PGR-A isoform in cyclic pigs, but not the truncated 5' UTR of the PGR-A isoform in pregnant pigs. This would suggest that different transcript variants of PGR-A exist in cyclic and pregnant endometrium.

The coding region common to PGR-B, PGR-A and the 5' UTR of PGR-C was detected by the AB2 primer pair. There was an effect of day on the abundance of the PGR-ABC isoform. PGR-ABC was present at high concentration on d 0 and d 5 and was decreased on d 7.5 in cyclic and d 12 in both cyclic and pregnant pigs. This pattern of expression most closely resembles the expression pattern of the PGR-B transcript assays by primer pair B1 in cyclic and pregnant pigs and primer pair B2 in cyclic pigs, although transcript abundance is not recovered by d 17. This suggests that another transcript variant (PGR-C) is being detected by this primer pair in addition to PGR-B and PGR-A, since the expression profile varies from the profile detected by the AB1 primer pair,

capable only of detecting PGR-B and PGR-A mRNA transcripts. Thus, the presence of PGR-C can be inferred by these results.

No significant variations in PGR mRNA abundance, however, were observed using the primer pair ABC, designed to assay total PGR expression. Expression patterns were likely not able to be detected because of the large standard errors. The large standard errors were caused by the wide variance in mRNA abundance between biological samples, although the variance remained low between technical replicates. Heterogeneous mixtures of endometrium cell types and necessitated detection of multiple isoforms that may be differentially regulated and expressed likely are the causes of the high variance.

The YWHAG transcript was a successful control gene for this study. Expression was expected to remain constant across days of the estrous cycle and early pregnancy based upon previous research (Kim, J.-G., 2007). While there were some minor fluctuations in abundance, expression of YWHAG remained did not significantly differ between cyclic and pregnant animals from d 0 – 17. The YWHAG transcript could thus be used as a control gene to increase confidence that variations in Ct values detected by qRT-PCR were due to enriched PGR mRNA abundance in the biological samples and not due to inconsistent amounts of total RNA assessed.

A caveat to these results is that there is a heterogeneous mixture of cell types within the endometrium. Previous studies indicate that PGR abundance may change not only with stage of the estrous cycle and/or early pregnancy, but also in a cell – specific manner in the endometrium. (Geisert et al., 1994; Kraus and Katzenellenbogen, 1993;

Tummaruk et al., 2009). As both the luminal and glandular epithelium are single cell layers, these cells contribute little to the overall sample content when compared with the stromal contribution. Additionally, each random sample of tissue used for RNA extraction will contain a unique combination of the different cell types. Finally, since the endometrium is manually separated from the myometrium, some contamination by myometrium and blood vessels is likely. Therefore, the wide biological variation observed in the qRT-PCR results may be real animal to animal variation or may be an artifact of the experimental technique. Also, the ability of this experiment to detect more distinct expression patterns may be compromised by dilution effects due to a heterogeneous mixture of cell types.

Finally, the presence of transcript splice variants has not been evaluated in pig mRNA. In humans, exons 4, 5 and 6 are all susceptible to excision by RNA splicing during transcript maturation (Misao et al., 1998; Marshburn et al., 2005). If similar splice variants exist in pig, their presence would bias the quantitation of total PGR detected by the ABC primer pair.

CONCLUSIONS

Day of the cycle or pregnancy affected the mRNA expression of PGR isoforms. Expression of PGR-B isoform mRNA was abundant on d 0 and d 5 and was decreased on d 7.5 in cyclic and d 15 in both cyclic and pregnant pigs. Expression of PGR-AB mRNA (measuring both the PGR-B and PGR-A transcripts) was low and constitutive on all days of pregnancy and the cycle except on d 15 when a transient surge in transcript abundance

occurred. The d 15 fold change induction of PGR-AB was nearly doubled in pregnant pigs versus cyclic pigs. There is evidence for the existence of a PGR-C isoform in porcine endometrial mRNA. Expression of PGR-ABC mRNA (measuring PGR-B, PGR-A and PBR-C transcripts) was abundant on d 0 and d 5 and was decreased on d 7.5 and d 12, with the other time points measured intermediate. The transcription initiation sites for PGR-B are located from 291 – 314 bases, with an alternative initiation site at 371 bp (GQ906379.1). Both encode the full-length PGR-B protein isoform. The transcription initiation site for PGR-A is located at 1046 bp (GQ903679.1) and encodes the full PGR-A protein isoform. Finally, there are 2 transcription initiation sites downstream of the PGR-A translation start codon at 2559 and 2631 bases that appear to encode PGR-C. The results from the qRT-PCR experiment would agree with the RPA data in suggesting that a PGR-C transcript does exist in porcine endometrium. Despite this evidence, the PGR-C isoform could not be observed on any of the Northern blots performed. It is possible that the PGR-C transcript is unstable and experimental conditions impair the ability to detect the PGR-C transcript consistently. Consequently, it is possible that the instability of the PGR-C in the living porcine uterus hinders its ability to be exported from the nucleus and translated into a function PGR-C protein.

CHAPTER V

CHARACTERIZATION AND LOCALIZATION OF PROGESTERONE RECEPTOR (PGR) PROTEIN IN PORCINE ENDOMETRIUM DURING THE ESTROUS CYCLE

ABSTRACT

The down-regulation of PGR within the luminal epithelium enables conceptus attachment and signaling within the porcine uterus. In humans, mice and rats three PGR mRNA isoforms (PGR-B, PGR-A and PGR-C) arise from alternative transcription start sites, encoding proteins with truncated N-termini that confer distinct biological functions to each alternative isoform. After determining the existence of PGR-B, PGR-A, and PGR-C mRNA in the porcine endometrium, the objective of this study was to both characterize and localize PGR protein expression during the estrous cycle. Three PGR antibodies were identified that were expected to cross-react with porcine PGR: a PGR-B-specific antibody and two PGR-AB antibodies which targeted the region of PGR protein common to PGR-B, PGR-A and PGR-C isoforms. The presence of PGR-B and PGR-A proteins was confirmed by immunoblotting, but none of the antibodies were able to detect PGR-C. Additionally, the molecular weights of the proteins identified, though consistent with previous findings in humans and pigs, were observed to be greater than predicted from amino acid sequence. Finally, abundance of PGR-B and PGR-A isoforms was

assessed on d 0, d 8 and d 12 (n = 3 samples per day), with no differences detected between the days. The three antibodies were then used for immunohistochemistry localization of PGR isoforms to specific cell types. The PGR-B specific antibody detected strong nuclear staining in the LE and uterine glandular epithelium (GE), decreasing in intensity from d 8 to d 12. Both PGR-AB antibodies, however, detected no nuclear staining in the LE and only sporadic staining of the stroma nuclei. Antibodies to PGR-AB also showed cytoplasmic staining in the uterine LE, GE and stroma that was not observed using the PGR-B antibody, with strongest staining on the apical surface of the LE on d 12. The PGR-A isoform, therefore, may have a more diffuse cytoplasmic staining than the strictly nuclear staining of PGR-B. Taken together, these results may indicate varying levels of biological activity rather than protein abundance of the two isoforms on days 8 and 12 of the estrous cycle in porcine endometrium.

INTRODUCTION

The uterus must undergo dynamic changes during both the establishment of pregnancy and during the estrous cycle, both in proliferation and differentiation of cells into deciduas (Larsen et al., 2002). In both uterine states, high, sustained concentrations of progesterone (P4) stimulate the rapid removal of progesterone receptor (PGR) from the uterine luminal epithelium (LE; Tseng and Zhu, 1997; Harduf et al., 2009; Kurita et al., 2000a, 2001; Mote et al., 1999; Arnett-Mansfeld et al., 2004; Spencer and Bazer, 2002).

This ligand induced down-regulation of PGR is an essential event for return to cyclicity in non-pregnant gilts and sows and permits attachment of the conceptuses in pregnant animals. Failure to achieve down-regulation of PGR could have severe repercussions, particularly in maintenance of pregnancy. Previous studies have demonstrated the disappearance of PGR from the LE and glandular epithelium (GE) in both the estrous cycle and in early pregnancy in other species, and the research presented here was based on experimental preliminary demonstration of this expression pattern specifically in pigs (Geisert et al., 2006; Ross et al., 2010).

In situ hybridization with a PGR-specific cRNA probe was used to localize PGR transcript expression at distinct time points during the estrous cycle and early pregnancy (Ross et al., 2010). Gilts were hysterectomized on d 0 (estrus), d 5, d 7.5, d 10, d 12, d 15 and d 17 of the estrous cycle and on d 10, d 12, d 15, and d 17 of early pregnancy. The results demonstrated the presence of PGR transcripts from days 0 to 5 in the LE, glandular epithelium (GE) and stroma, with rapid loss in both pregnant and cyclic gilts as serum P4 concentrations rose due to CL production until complete depletion by day 13 in the LE and GE. The LE and GE expression of PGR recovered from undetectable amounts beginning d 15 and continuing to d 17 in cyclic gilts, concurrent with regression of the CL. The PGR mRNA expression in the stroma remained constant among treatments (Ross et al., 2010). These results demonstrate the dynamic changes in PGR mRNA expression in the porcine endometrium with implications for fertility and maintenance of pregnancy in pigs.

The PGR protein exists in several different isoforms in humans, mice and rats that arise from different transcripts encoding proteins with N-terminus deletions. In humans,

the PGR-B isoform is 933 amino acid residues in length and its molecular weight is 116 kDa (Wei et al., 1996). The PGR-A isoform is a deletion of the N-terminal 164 amino acid residues and has a molecular weight of 96 kDa. The PGR-C isoform is a further N-terminus truncation and has a molecular weight of 60 kDa (Wei et al., 1996). Each of the three isoforms confers distinct biological functions. These isoforms have not yet been characterized or localized in porcine endometrium. The spatial and temporal-specific expression of the different isoforms may play an important role in uterine receptivity to pregnancy and in control of the estrous cycle.

The results of Ross (2006) do not attempt to differentiate between variant PGR isoforms, which may be regulated independently, have different signaling pathways and confer different biological activities. The study also does not investigate PGR expression at the level of protein or protein functionality, neither of which is necessarily concordant with fluctuations in PGR mRNA transcription. Further study is required to elucidate the patterns of expression specific to each isoform in the hope of more comprehensive understanding of the role of PGR in cyclicity and pregnancy.

MATERIALS AND METHODS

Samples

Uteri from gilts on d 0 (observed estrus) and gilts on d 8 and d 12 after insemination on d 1 were collected by hysterectomy (n = 3 / day) at the University of Missouri – Columbia. Uterine horns were dissected and endometrium (5-10 g) was

manually removed from the myometrium. Samples were immediately flash frozen in liquid nitrogen and stored at -80°C.

Protein isolation

Frozen endometrial samples were ground into powder in liquid nitrogen. For protein isolation, 0.6 grams of powdered tissue was removed and homogenized in a lysis buffer of 50 mM Tris, 50 mM dithiothreitol, 150 mM sodium chloride, 2% sodium dodecyl sulfate and 20% glycerol. Samples were centrifuged at 13,000 rpm for 10 minutes. Supernatant was removed and sonicated. Protein concentration was quantified in two ways: by NanoDrop-1000 (NanoDrop Technologies, Wilmington, DE) and using Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL). The BCA Kit colorimetric assay proved more accurate and was used to determine protein concentration of samples to be used for subsequent assays.

Western Blot

Western Blot protocol was adapted from protocols from Invitrogen (Carlsbad, CA), GE Healthcare (Buckinghamshire, UK) and Cell Signaling Technology, Inc. (Danvers, MA) and optimized. Fifty µg of each protein sample was combined with 1 µL Reducing Agent and 2.5 µL 4X LDS Sample Buffer (Invitrogen, Carlsbad, CA) and boiled for 10 minutes to denature the proteins. Total protein was separated by molecular

weight on a NuPage® Novex Bis-Tris 10% Gel (Invitrogen, Carlsbad, CA) with PageRuler™ Prestained Protein Ladder (Fermentas Inc., Glen Burnie, MD) at 100V until sufficient separation of the ladder (approximately 8 hours). Size-separated proteins were transferred to a PVDF membrane using 1X Transfer Buffer (Invitrogen, Carlsbad, CA) at 4°C and 50V for 2 hours and 100 V at room temperature for 30 minutes. The membrane was then incubated with 2% w/v Amersham ECL Blocking Agent (GE Healthcare, Buckinghamshire, UK) in TBS-T for 1 hour. The membrane was placed in a solution of the primary antibody in a sealed humidity chamber overnight. The next day, the membrane was washed thoroughly in TBS-T for approximately 45 minutes. The membrane was then incubated in a secondary antibody blocking solution consisting of 2% w/v Amersham ECL Blocking Agent (GE Healthcare, Buckinghamshire, UK) in TBS-T and a 1:10,000 dilution of either anti-mouse or anti-rabbit antibody for 1 hour on a shaker. The membrane was washed thoroughly for approximately 45 minutes in TBS-T, then held in fresh TBS-T for detection. In a dark room, Amersham ECL Detection Kit chemiluminescent detection solution was added to the membrane for five minutes, and then the excess drained. The membrane was exposed to BIOMAX™ MS film (Eastman Kodak Co., Rochester, NY) and developed. The film was scanned into a computer as a .jpeg file and band intensity analyzed using default settings of the publicly-available ImageJ software (U.S. National Institutes of Health, Bethesda, MA).

Four primary antibodies were used for Western Blot assays. The first antibody was raised against a peptide sequence specific to PGR-B in rabbit (Cell Signaling Technologies Inc., Danvers, MA) which could exclusively detect the PGR-B isoform. The antibody is raised to the residues surrounding amino acid residue Ser 115. The

dilution factor of this antibody for overnight incubation was 1:1,000 in TBS-T with 5% w/v Bovine Serum Albumin as a blocking agent to prevent non-specific antigen binding. The second antibody (Millipore PGR-AB) had been validated to detect PGR-B and PGR-A in human. The antibody was raised in mouse against the region of human PGR common to PGR-B, PGR-A and PGR-C (Millipore, Billerica, MA). The dilution factor of this antibody for overnight incubation was 1:50 in TBS-T with 5% w/v Bovine Serum Albumin as a blocking agent to prevent non-specific antigen binding. The third antibody (Invitrogen PGR-AB) is a mouse anti-PGR validated to detect human PGR-B and PGR-A (Invitrogen, Carlsbad, CA) and directed against the amino acid residues around Tyr 541. The dilution factor of this antibody for overnight incubation was 1:50 in TBS-T with 5% w/v Bovine Serum Albumin as a blocking agent to prevent non-specific antigen binding. All PGR antibodies were unconjugated and monoclonal. A rabbit polyclonal antibody to alpha tubulin predicted to cross-react with pig α-tubulin protein was used as a loading control for protein samples (Abcam Inc., Cambridge, MA). The dilution factor of this antibody for overnight incubation was 1:50 in TBS-T with 5% w/v Bovine Serum Albumin as a blocking agent to prevent non-specific antigen binding.

The data were analyzed as a completely randomized experimental design with day (0, 8, 12) as the independent variable. Samples were normalized for total protein concentration by calculating the ratio of each isoform (Cell Signaling PGR-B, Millipore PGR-B, Millipore PGR-A and Invitrogen PGR-B) intensity determined by ImageJ software to the intensity of loading control protein, α-tubulin. The normalized intensities were analyzed as the dependent variables using the general linear model procedure (Proc GLM) of the Statistical Analysis System software (SAS Institute Inc., Cary, N.C.). The

effect of day was estimated on the intensity of 4 PGR isoforms (Cell Signaling PGR-B, Millipore PGR-B, Millipore PGR-A and Invitrogen PGR-B). Least squares means and pooled standard errors (SEM) were calculated using the LSMEANS procedure of SAS. The Levene test (HOVTEST) statement was used to test for homogeneity of variances under the means statement of SAS. Variances did not significantly differ for any of the normalized isoform intensities analyzed (Cell Signaling PGR-B: $P = 0.11$; Millipore PGR-B: $P = 0.14$; Millipore PGR-A: $P = 0.21$; Invitrogen PGR-B: $P = 0.12$).

Immunohistochemistry

Cellular localization of PGR protein by immunohistochemistry was performed on paraffinized cross-sections (5 µm) of porcine uterine horns harvested on d 8 or d 12 of inseminated gilts (d 0 = estrus; n = 4 animals / day). The VECTASTAIN® *Elite*® ABC Kit (Vector Laboratories, Inc., Barlingame, CA), an immunoperoxidase-based method of detection was used and the protocol optimized for laboratory conditions. To deparaffinize and hydrate samples, slides were submerged and constantly agitated in xylene twice for 6 minutes each. Slides were then submerged twice in 100% ethanol solutions for 3 minutes each, followed by one immersion in 95% ethanol for 1 minute and one immersion in 85% ethanol for 1 minute. Slides were submerged 30 – 50 times in deionized water and transferred to a 1X PBS bath.

Antigen retrieval was achieved by boiling slides in 0.01 M citrate buffer in the microwave for approximately 5 minutes. Slides were then transferred to ice and cooled for 30 minutes and returned to 1X PBS. Slides were incubated in 0.3% hydrogen peroxide for 30 minutes at 25°C to quench endogenous peroxidase activity and washed

thoroughly in 1X PBS for 5 minutes. Excess fluid was removed and samples were enclosed on slides using a wax pen.

Sections were incubated with normal blocking serum (VECTASTAIN®) to control non-specific binding of the primary antibody for 30 minutes and then serum removed. Then sections were incubated at 4°C overnight in a humidity chamber with the primary antibody (PGR-B, Millipore PGR-AB, or Invitrogen Millipore PGR-AB). In the morning, slides were again washed rigorously for 5 minutes in 1X PBS at room temperature. Sections were incubated next with the avidin: biotinylated peroxidase conjugated secondary antibody (VECTASTAIN®, either anti-mouse for Invitrogen or Millipore PGR-AB primary antibodies or anti-rabbit for PGR-B primary antibody) for 30 minutes and washed again for 5 minutes in 1X PBS before a 30 minute incubation with ABC Reagent (VECTASTAIN®). Slides were washed for 5 minutes in 1X PBS, then incubated with ImmuPact DAB (Vector Laboratories, Inc., Barlingame, CA) an HRP substrate for 5 minutes to develop the brown color. After a 5 minute rinse in deionized water, slides were immersed into a 1:3 dilution of haematoxylin as a counterstain for 40 seconds, rinsed gently in running tap water, immersed for 1 minute in Scott's Bluing Reagent and gently rinsed again under running tap water for 2 minutes. Slides were then dehydrated using one 70% ethanol bath for 2 minutes, two 95% ethanol baths for 2 minutes each, two 100% ethanol baths for 5 minutes each and two baths in fresh xylene for 10 minutes each. Coverslips were added using Permount and allowed to dry before examination under a microscope.

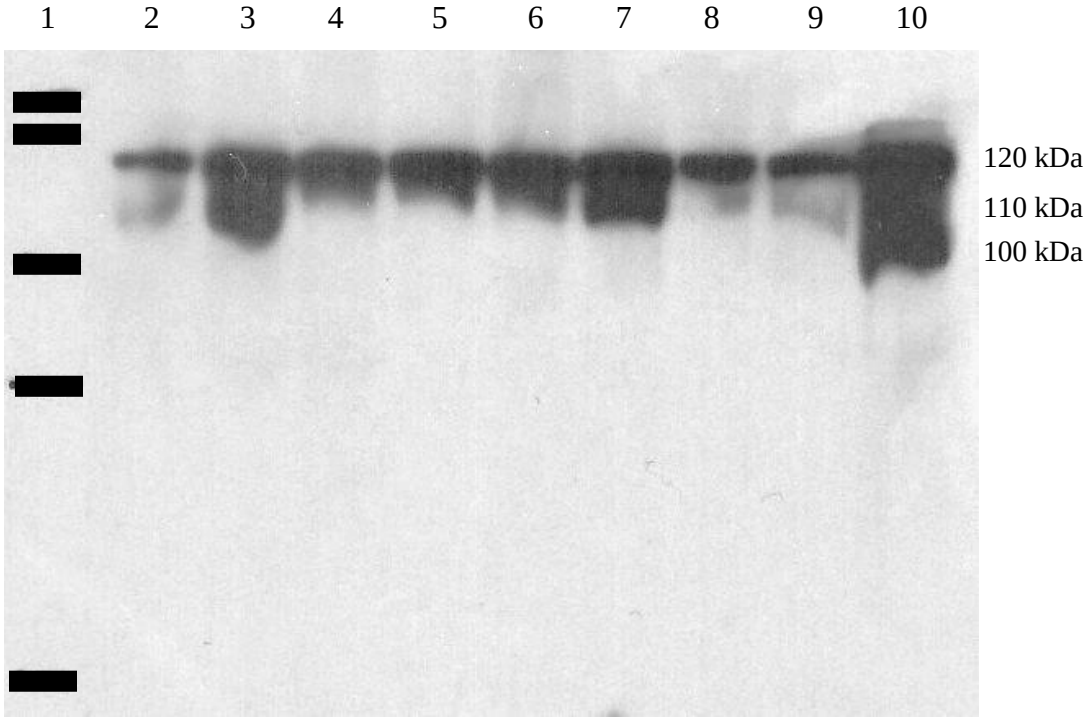
Three primary antibodies were used for IHC. The first antibody (PGR-B) was specific to PGR-B raised in rabbit (Cell Signaling Technologies Inc., Danvers, MA). The

antibody is raised to the residues surrounding amino acid residue Ser 115. The dilution factor of this antibody for overnight incubation was 1:200 in 1X PBS. The second antibody (Millipore PGR-AB) was validated to detect porcine PGR-B and PGR-A by Western Blot in this study. The antibody was raised in mouse against the region of human PGR common to PGR-B, PGR-A and PGR-C (Millipore, Billerica, MA). The dilution factor of this antibody for overnight incubation was 1:40 in 1X PBS. The third antibody (Invitrogen PGR-AB) was a mouse anti-PGR validated by Western Blot in this study to detect porcine PGR-B and PGR-A (Invitrogen, Carlsbad, CA). The antibody is raised to the residues surrounding amino acid residue Tyr 541. The dilution factor of this antibody for overnight incubation was 1:50 in 1X PBS. All antibodies were unconjugated and monoclonal. Slides were assessed and documented using a Leica light microscope at both 100X and 400X magnification.

RESULTS

Different PGR isoforms are present in the endometrium of pigs at various phases of the estrous cycle. Immunoblotting was used to demonstrate the presence of two PGR isoforms. Using a PGR-B specific antibody, one major band consistently present at 120 kDa molecular weight in all samples with 2 secondary bands to 110 and 100 kDa observed in some individual samples (Figure 5.1) were detected by Western blot analysis. Using 2 different PGR-A/B antibodies designed to the region common to B, A and C isoforms, Western Blot analysis detected 2 bands present for both of the antibodies. One

Figure 5.1 Protein abundance of PGR in porcine endometrial cells on d 0, d 8 and d 12 of the estrous cycle assessed by Western blot with antibody specific to PGR-B isoform. Equal amounts of protein (50 µg) isolated from lysed endometrial cells were size-separated by electrophoresis in each lane. Lanes contain: 1 = PageRuler (top to bottom 170 kDa, 130 kDa, 95 kDa, 72 kDa and 55 kDa, with migration locations of products manually recorded); 2-4 = d 0 (estrus); 5-7 = d 8; 8–10 = d 12 samples. The band at 120 kDa represents the dominant PGR-B protein with a less intense PGR-B protein variant at 110 kDa present in lanes 2 and 6 and 100 kDa variant present in Lane 10. Abundance of protein does not differ significantly with day of the estrous cycle.



of these bands localized to 120 kDa, and the other migrated at a molecular weight of 96 kDa (Figure 5.2 and 5.3). PGR-C isoform is expected to migrate at approximately 60 kDa molecular weight. A band of this size was not present in immunoblots using any of the 3 antibodies. A few shadow bands, however, were detected by the antibodies: one at about 10 kDa smaller than the 120 kDa major PGR-B band (110 kDa), one about 20 kDa smaller than the 120 kDa major PGR-B band (100 kDa) and one about 10 kDa smaller than the 96 kDa major PGR-A band.

An antibody to alpha tubulin was expected to remain constant across the estrous cycle and was used as a loading control to ensure equal amounts of protein were being loaded for each sample (Figure 5.4). Normalized intensities for each isoform assayed were analyzed for the effect of day on protein abundance. No significant differences in expression were observed on d 0, d 8 and d 12 of the estrous cycle for the PGR-B antibody ($P = 0.32$; Figure 5.1). Using either the Millipore or the Invitrogen PGR-A/B antibody, the PGR-A isoform present at 96 kDa was less abundant than the PGR-B band at 120 kDa (Figure 5.2 and 5.3). Abundance of PGR-B (120 kDa) detected by Millipore and Invitrogen PGR-AB antibodies was significantly greater in d 0 (estrus) samples than in d 8 and d 12 samples (both $P < 0.05$). Abundance of PGR-A (96 kDa) detected by the Millipore antibody did not differ significantly with day of the estrous cycle ($P = 0.14$). Abundance of PGR-A (96 kDa) by the Invitrogen antibody could not be quantified using ImageJ software.

Figure 5.2 Protein abundance of PGR in porcine endometrial cells on d 0, d 8 and d 12 of the estrous cycle assessed by Western blot with Millipore antibody against PGR-B, PGR-A and PGR-C common sequence. Equal amounts of protein (50 µg) isolated from lysed endometrial cells were size-separated by electrophoresis in each lane. Lanes contain: 1 = PageRuler (top to bottom 170 kDa, 130 kDa, 95 kDa, 72 kDa, with migration locations of products manually recorded); 2-4 = d 0 (estrus); 5-7 = d 8; 8–10 = d 12 samples. The band at 120 kDa represents the dominant PGR-B protein with a less intense PGR-B protein variant at 110 kDa present in selected samples. The band at 96 kDa represents the PGR-A protein. The PGR-B isoform appears to be consistently more abundant than the PGR-A isoform. The PGR-B isoform is more abundant on d 0 of the estrous cycle than d 8 and d 12 ($P < 0.05$), while PGR-A did not differ significantly between days.

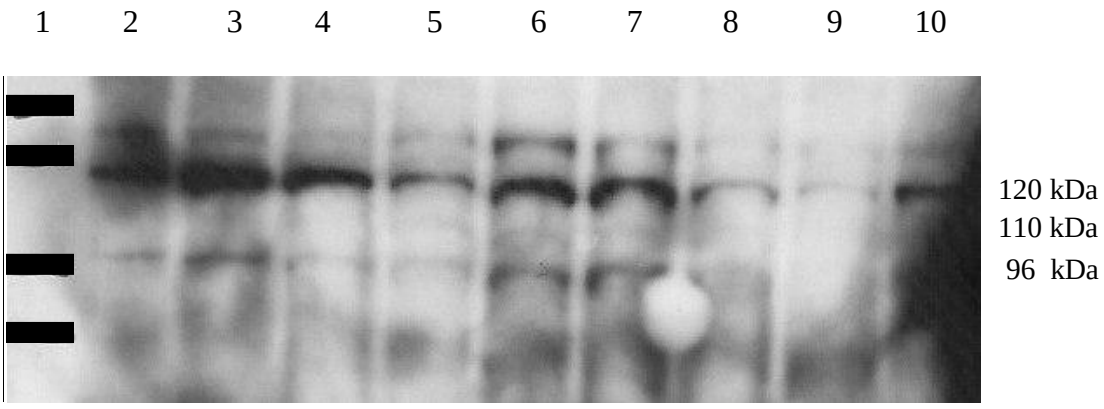


Figure 5.3 Protein abundance of PGR in porcine endometrial cells on d 0, d 8 and d 12 of the estrous cycle assessed by Western blot with Invitrogen antibody against PGR-B, PGR-A and PGR-C common sequence. Equal amounts of protein (50 µg) isolated from lysed endometrial cells were size-separated by electrophoresis in each lane. Lanes contain: 1 = PageRuler (top to bottom 130 kDa, 95 kDa and 72 kDa with migration locations of products manually recorded); 2-4 = d 0 (estrus); 5-7 = d 8; 8–10 = d 12 samples. The band at 120 kDa represents the dominant PGR-B protein with a less intense PGR-B protein variant at 110 kDa present in selected samples. The band at 96 kDa represents the dominant PGR-A protein with a less intense PGR-A variant at 83 kDa in selected samples. The PGR-B isoform appears to be consistently more abundant than the PGR-A isoform. The PGR-B isoform is more abundant on d 0 of the estrous cycle than on d 8 or d 12 ($P < 0.05$). The PGR-A isoform, however, was not able to be quantified.

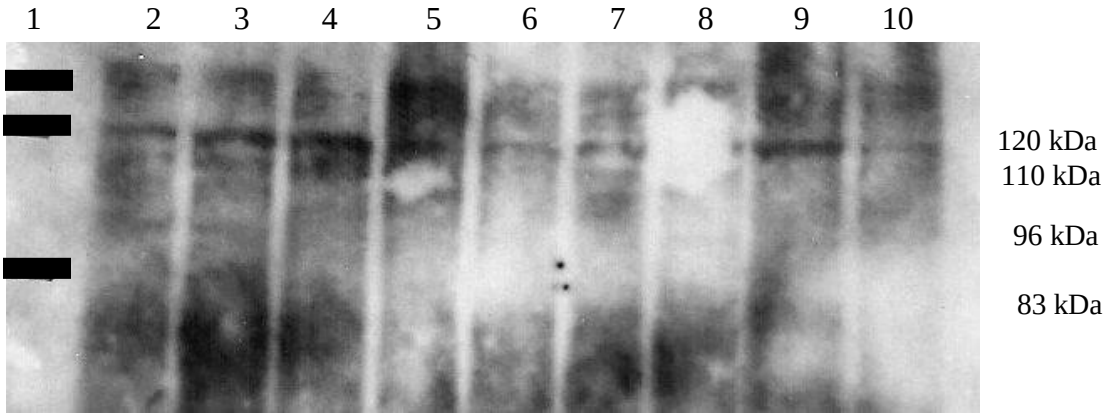
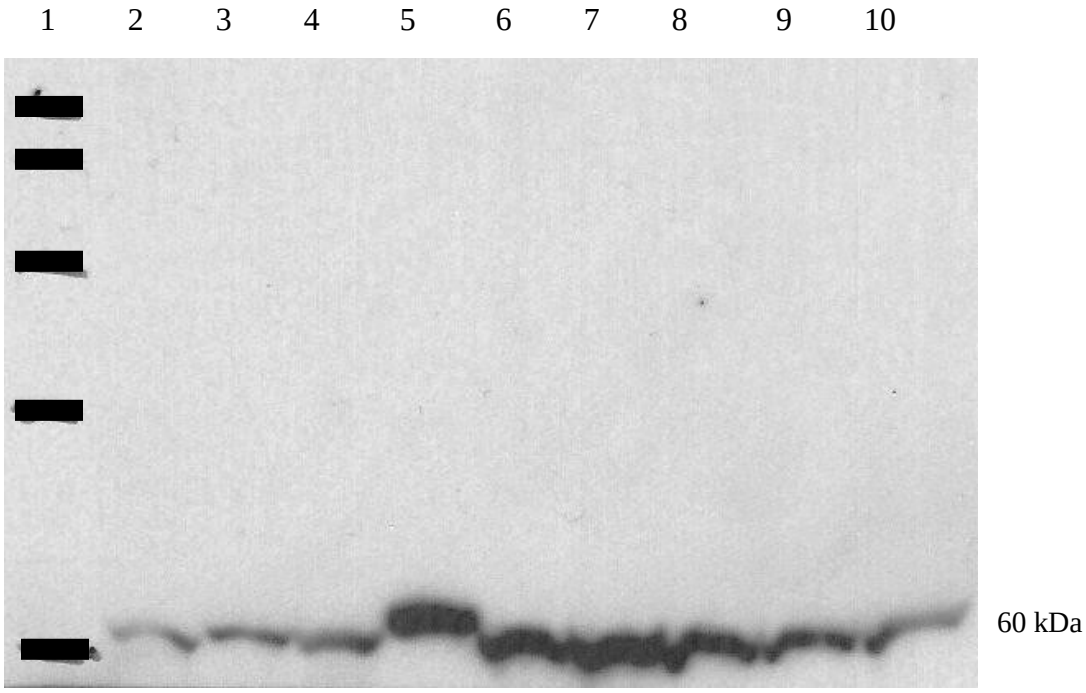


Figure 5.4 Protein abundance of loading control protein α-tubulin in porcine endometrial cells on d 0, d 8 and d 12 of the estrous cycle assessed by Western blot. Equal amounts of protein (50 µg) isolated from lysed endometrial cells were size-separated by electrophoresis in each lane. Lanes contain: 1 = PageRuler (top to bottom 170 kDa, 130 kDa, 95 kDa, 72 kDa and 55 kDa with migration locations of products manually recorded); 2-4 = d 0 (estrus); 5-7 = d 8; 8–10 = d 12 samples. Porcine α-tubulin has a molecular weight of 60 kDa.



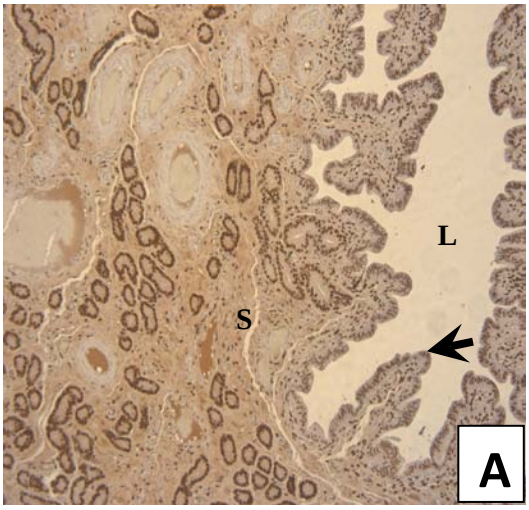
Immunohistochemistry was used to localize PGR isoforms to specific cell types within the endometrium. The PGR-B specific antibody demonstrated strong nuclear staining in the nuclei of GE and LE on d 8 (Figure 5.5). Expression of PGR-B in LE nuclei is decreased from d 8 to d 12 but appears to remain constant in the GE from d 8 to d 12. Using the PGR-B specific antibody no staining of the cytoplasm was observed on either d 8 or d 12. Staining of the stroma nuclei was sporadic. The Millipore PGR-AB antibody demonstrated no nuclear staining in the LE or GE on either d 8 or d 12 of the estrous cycle (Figure 5.6). Strong cytoplasmic staining was observed in the LE and GE on d 8 and decreased cytoplasmic staining on d 12. Immunostaining was localized to the apical surface of the LE on d 12. Sporadic staining of the stroma nuclei was observed on d 8 with decreased intensity on d 12. The Invitrogen PGR-AB antibody demonstrated no nuclear staining in the LE or GE on either d 8 or d 12 of the estrous cycle (Figure 5.7). Nuclear staining was observed in GE on d 8 and d 12. Strong cytoplasmic staining was observed in the LE and GE on d 8 and decreased cytoplasmic staining on d 12. Immunostaining was localized to the apical surface of the LE on d 12. Sporadic staining of the stroma nuclei was observed on d 8 with decreased intensity on d 12. None of the staining patterns was due to non-specificity of either the primary or secondary antibodies, as determined by negative controls performed on endometrial samples (Figure 5 .8).

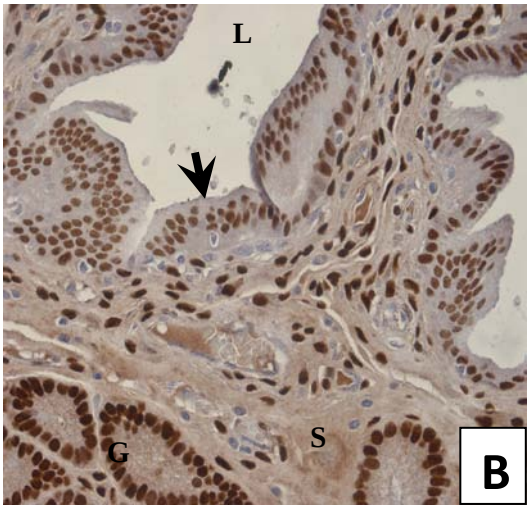
Figure 5.5 Cell type-specific immunohistochemical localization of PGR in porcine endometrium paraffin sections on d 8 and d 12 of the estrous cycle with antibody specific to PGR-B protein. Panel A: representative section of d 8 endometrium at 100X magnification, B: same section at 400X magnification, C: representative section of d 12 endometrium at 100X magnification, D: same section at 400X magnification. Structures shown are: L = uterine lumen; S = endometrial stroma; G = uterine gland; arrow = luminal epithelium. Images were taken with a Leica light microscope. Strong nuclear localization observed in LE on d 8 of the estrous cycle with decreased intensity on d 12. Strong nuclear localization observed on d 8 and d 12 in GE, with sporadic staining of stroma nuclei on both days. No cytoplasmic staining observed.

100X Magnification

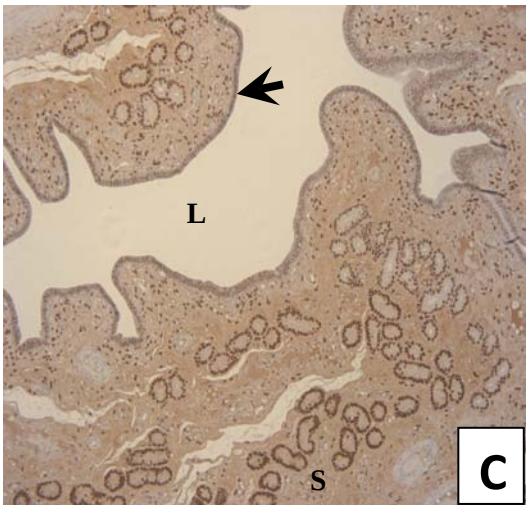
400X Magnification

d





d12



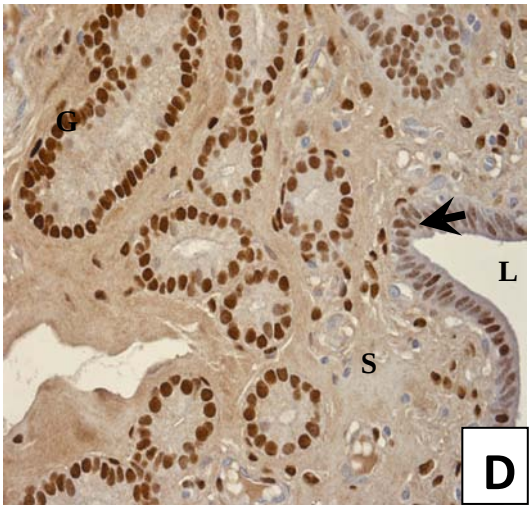
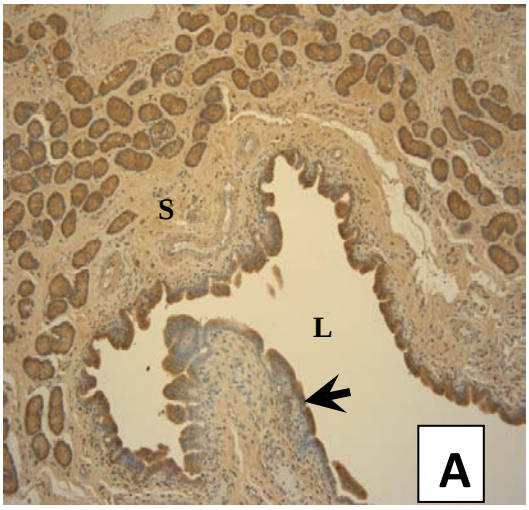


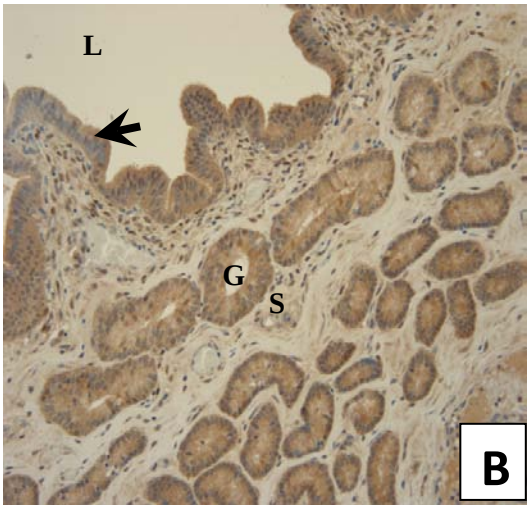
Figure 5.6 Cell type-specific immunohistochemical localization of PGR in porcine endometrium paraffin sections on d 8 and d 12 of the estrous cycle with Millipore antibody raised against the PGR-B, PGR-A, PGR-C common region. Panel A: representative section of d 8 endometrium at 100X magnification, B: same section at 400X magnification, C: representative section of d 12 endometrium at 100X magnification, D: same section at 400X magnification. Structures shown are: L = uterine lumen; S = endometrial stroma; G = uterine gland; arrow = luminal epithelium. Images were taken with a Leica light microscope. Nuclear staining not observed on d 8 or d 12 in LE and GE. Strong cytoplasmic staining present in LE and GE on d 8. Lower intensity cytoplasmic staining observed on d 12, with localization to the apical surface of LE. Sporadic nuclear staining of stroma on d 8 and slightly less on d 12.

100X Magnification

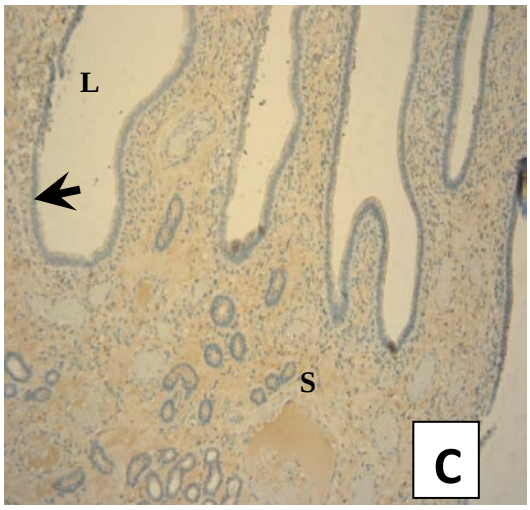
400X Magnification

d





d12



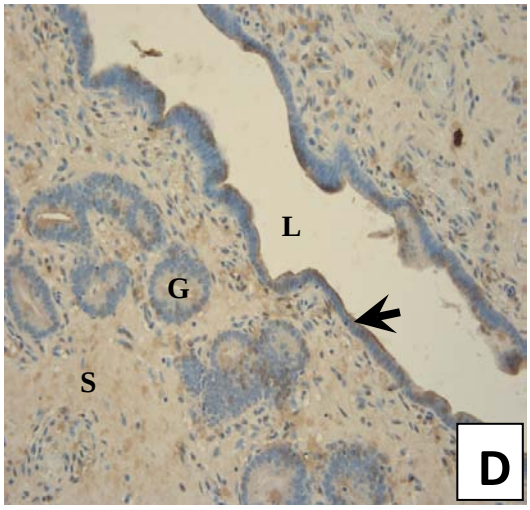
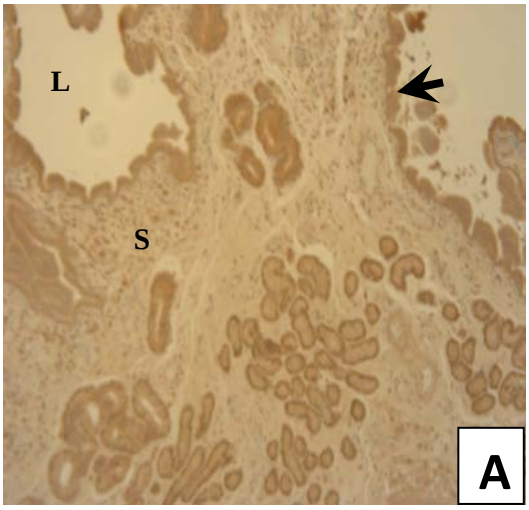


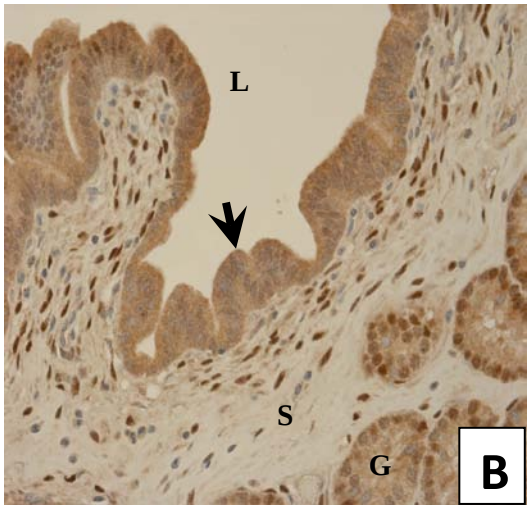
Figure 5.7 Cell type-specific immunohistochemical localization of PGR in porcine endometrium paraffin sections on d 8 and d 12 of the estrous cycle with Invitrogen antibody raised against the PGR-B, PGR-A, PGR-C common region. Panel A: representative section of d 8 endometrium at 100X magnification, B: same section at 400X magnification, C: representative section of d 12 endometrium at 100X magnification, D: same section at 400X magnification. Structures shown are: L = uterine lumen; S = endometrial stroma; G = uterine gland; arrow = luminal epithelium. Images were taken with a Leica light microscope. Nuclear staining not observed on d 8 or d 12 in LE. Nuclear localization observed in GE on d 8 and d 12. Strong cytoplasmic staining present in LE and GE on d 8. Lower intensity cytoplasmic staining observed on d 12, with localization to the apical surface of LE. Sporadic nuclear staining of stroma on d 8 and slightly less on d 12.

100X Magnification

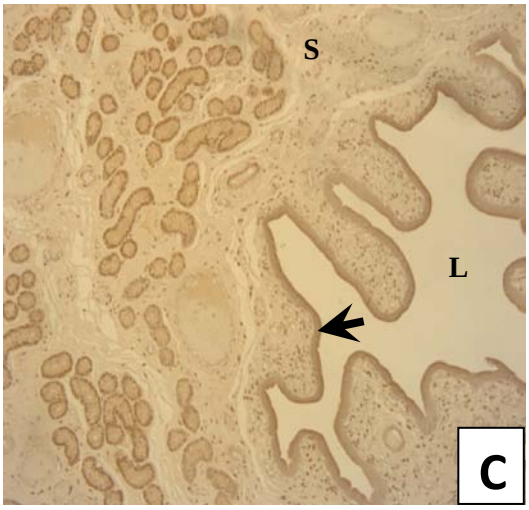
400X Magnification

d





d12



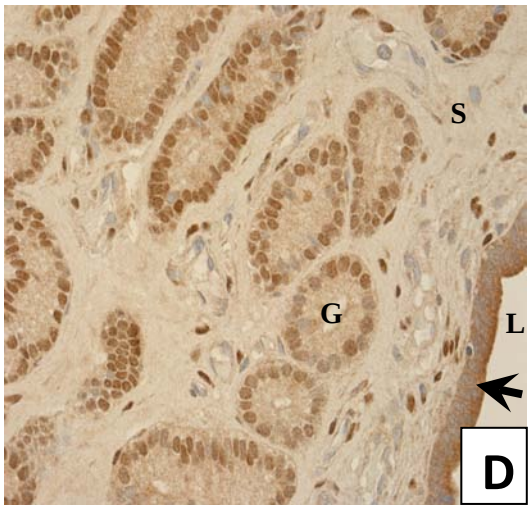
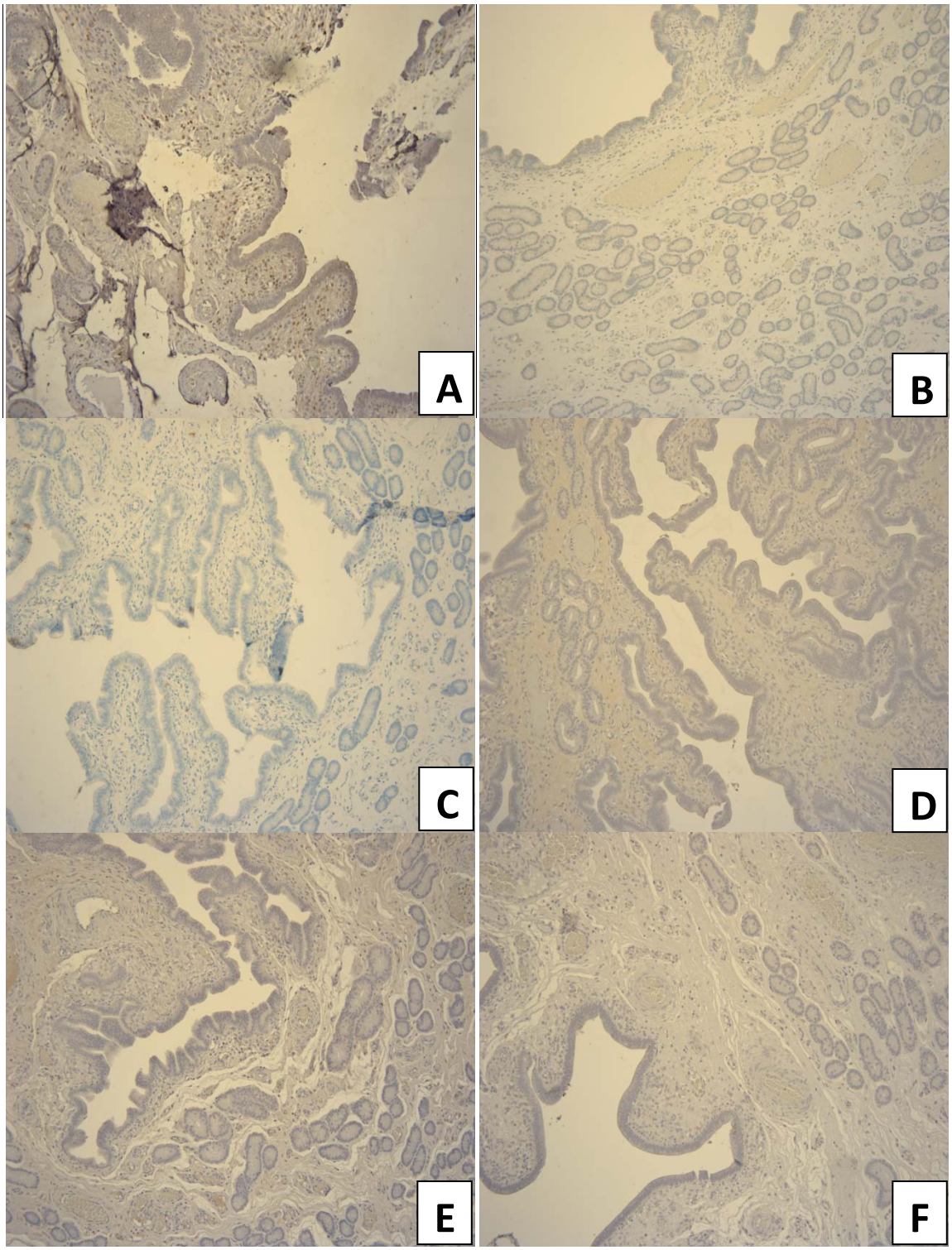


Figure 5.8 Negative control slides for immunohistochemical localization of PGR in porcine endometrium. Panel A: primary antibody negative, anti-rabbit secondary antibody positive, B: primary antibody negative, anti-mouse secondary antibody positive, C: PGR-B primary antibody positive, secondary antibody negative, D: Millipore PGR-A/B primary antibody positive, secondary antibody negative, E: Invitrogen PGR-A/B primary antibody positive, secondary antibody negative, F: primary antibody negative, secondary antibody negative. All images were taken at 100X magnification with a Leica light microscope.


A
B
C
D
E
F

DISCUSSION

Western Blot assay could not verify the presence of a PGR-C protein. PGR-C was not detected by either of 2 antibodies capable of detecting it (Millipore PGR-AB and Invitrogen PGR-AB), lending credence to the conclusion that it is truly not present in the protein milieu at detectable levels. It is possible, therefore, that the PGR-C isoform detected by Northern Blot and RPA in Chapter 4 at the mRNA level is not being translated into protein in this biological system. Another possibility is that the PGR-C isoform is, indeed translated into a corresponding protein isoform but is so rapidly degraded that it was undetectable by immunoblotting. PGR-C mRNA may also be an artifact of the Northern Blot and RPA techniques and not actually biologically relevant. The final possibility is that the conformation of PGR-C is distinct from PGR-B or PGR-A and was unavailable to the antibody for detection. This, however, seems unlikely as the proteins were run on a denaturing gel that should have eradicated secondary protein structures and obscuring native conformations. Therefore, we conclude it is likely that PGR-C is not present at the protein level in the cyclic porcine endometrium from d 0 through d 12.

Analysis of Western Blot results also revealed that the molecular weight of the PGR proteins was greater than the expected molecular weight predicted from amino acid sequence. Sequence prediction expects PGR-B to be 99 kDa and PGR-A to be 83 kDa. Additionally, three light shadow bands can be identified on the Western Blots corresponding to the major bands. Taken together, these results might suggest post-translational modifications to the PGR protein. Since these modifications would be

covalently bound to the amino acid residues, they would not be expected to be removed by the denaturation process. In human PGR, three lysine residues (Lys 7, Lys 388 and Lysw531) have been identified as targets of SUMOylation. Small ubiquitin-like modifier (SUMO) is a small protein 100 amino acid residues in length with a 12 kDa molecular weight which covalently binds to lysine residues. SUMO has been demonstrated in PGR to increase transcriptional activity of PGR-targeted genes. Other nuclear receptor superfamily members are known to be targets of SUMOylation, as well (Poukka et al., 2000; Tian et al., 2002; Sentis et al., 2005). PGR-B would be the only isoform to contain the Lys 7 residue to be modified. Both PGR-B and PGR-A contain Lys 388 which may also be SUMOylated. The lower-abundance bands to 100 kDa and 110 kDa may correspond to unmodified PGR-B and PGR-B with one SUMO modification, respectively, with the highly abundant 120 kDa band representing PGR-B with both Lys 7 and Lys 388 SUMOylated. PGR-A, likewise may have a lowly abundant unmodified band at 85 kDa, with a more highly abundant band corresponding to PGR-A plus one SUMO modification at Lys 388 at 96 kDa molecular weight. Indeed, SUMO has been demonstrated to enhance the intranuclear localization of proteins and to stabilize these proteins (Kurepa et al., 2003), so that the higher abundance of highly SUMOylated PGR proteins may indicate their localization into the nucleus and high activity there as transcription factors.

Using immunohistochemistry, PGR-B was localized strongly to the nucleus of the LE and GE, as expected. PGR-B abundance also decreases from d 8 to d 12 of the cycle, as expected. However, expression of PGR-A was unexpected. Both Millipore and Invitrogen PGR-AB antibodies failed to detect any nuclear PGR in the LE on either d 8

or d 12. This is unexpected because this antibody should detect everything detectable with the PGR-B antibody, in addition to potentially detecting the presence of PGR-A in cellular locations where PGR-B is absent. Neither antibody, however, could recapitulate the strong nuclear staining in the LE detected by the PGR-B antibody. The PGR-AB antibodies both detected sporadic nuclear staining in the stroma of the endometrium and the Invitrogen PGR-AB detected PGR protein in the nucleus of GE on d 8 and d 12, indicating that the antibodies were capable of entering and staining the nucleus, The cytoplasmic staining of the LE, GE and stroma detected with the first Invitrogen PGR-AB antibody was validated by the second Millipore PGR-AB antibody, suggesting that this result was not an antibody artifact or non-specific staining. Since no cytoplasmic staining was detected with the PGR-B specific antibody, we conclude that PGR-A may show more diffuse expression in the cytoplasm of GE and LE and throughout the stroma. Localization of PGR to the cytoplasm of cells has been observed previously in PGR (Jacobs et al., 1980; Berkquist et al., 1981).

It is possible to speculate that the presence of PGR-A in the cytoplasm may have implications for its biological activity in the endometrium. Since PGR is a nuclear transcription factor, it may not be biologically active if sequestered to the cytoplasm, allowing PGR-B to be the dominant active isoform in the endometrium even in the presence of PGR-A transcripts and proteins in the whole cell milieu.

Finally, PGR-A appears to localize to the apical surface of the LE adjacent to the uterine lumen. This pattern of expression is distinct from its cytoplasmic localization in the GE or the stroma. This localization pattern was observed using both PGR-A/B antibodies, decreasing the probability that the observed expression was a spurious result.

While unexpected for PGR, there is an established method of directional secretion by the LE cells. During pregnancy, $PGF_{2\alpha}$ switches from being secreted into the uterine vasculature so that it can initiate luteolysis to being secreted into the uterine lumen, thereby protecting the CL from degradation (Ziecik, 2002; Bowen et al., 1996; Diaz and Wiltbank, 2004). There is also evidence that the side of exposure to a stimulus determines the direction of secretion from the LE of pigs (Braileanu et al., 2000). Taken together, this suggests that a product within the uterine lumen may be stimulating the apical surface of the LE specifically to transport PGR-A toward the apical surface and potentially to secrete PGR-A into the lumen. Stroma cells do not exhibit polarity, so they would be incapable of establishing directional secretion, and glandular epithelial cells, while polarized, would not be exposed to stimulating product, if it were confined within the uterine lumen. This might explain why we see this distinct expression pattern in the LE but not the other cell types, although they do express PGR-A in the cytoplasm.

CONCLUSIONS

In the porcine endometrium, PGR-B and PGR-A isoforms can be detected. The molecular weight of the PGR-B protein in pigs is approximately 120 kDa. The molecular weight to which PGR-A migrates in pigs is approximately 96 kDa. Both PGR isoforms may be modified with SUMO proteins covalently bound to the PGR protein in vivo. PGR-B contains 2 SUMO binding sites. The dominant band migrating to 120 kDa may, therefore, have 2 SUMO proteins covalently associated with PGR-B. Only 1 of these

sites is present in PGR-A, so that 1 covalently bound SUMO protein may result in the dominant 99 kDa band. Given that SUMO assists cells with nuclear localization and can stabilize proteins, SUMOylation may be a key event in PGR protein maturation and functionality.

Concentrations of PGR-B was increased on d 0 over d 8 or d 12, but protein presence was not ablated. This was not entirely expected. Many studies have observed PGR mRNA decreased by d 7 and very low to undetectable by d 11 – 12. This is evidence that mRNA abundance and protein abundance may be uncoupled in the PGR system. The mRNA molecules may be translated more than once, resulting in a more constitutive level of PGR protein, despite the decrease in transcription. Another possibility is that PGR protein degradation is abrogated during the period that transcription is decreased. An interesting question to address in the future is whether the constant level of protein results in constant levels of PGR transcription factor activity on progesterone-responsive genes.

Nuclear staining of PGR-B was observed in the LE and GE of both d 8 and d 12 porcine endometrium. This was expected, as was the decrease in staining from d 8 to d 12. The result, however, is inconsistent with the results of the Western Blot analysis which did not demonstrate a difference in abundance of PGR-B in total endometrial cell proteins. One confounding factor of the Western Blot experiment is that endometrium samples are a heterogeneous mixture of cells (LE, GE, stroma, vascular cells and perhaps some stray myometrial cells). The variation in composition of each sample may obscure our ability to detect changes in the PGR-B and PGR-A expression patterns during the estrous cycle.

Also unexpected was the apparent inability of the PGR-AB common region antibodies to bind PGR-B in endometrium tissue sections. The antibodies were able to detect both isoforms on a Western Blot, indicating that both are capable of binding denatured PGR-B. Given that the native conformation of PGR-B does not appear to be recognized by these antibodies, the ability of the antibodies to recognize cytoplasmic PGR-A *in vivo* suggests that PGR-A exists in a different conformation than PGR-B in vivo. This may indicate that PGR-A is not functionally active in endometrial cells on d 8 and d 12 of the estrous cycle. It will be interesting to evaluate the presence of PGR-A detected by these two antibodies at different time points during the estrous cycle, and also to use qRT-PCR to measure the expression of known PGR-A responsive genes. Together, these may address the question of PGR-A functionality.

The PGR-A isoform was expressed in the cytoplasm of LE and GE and intermittently in the nucleus of stroma cells. Additionally, cytoplasmic presence of PGR-A in the LE was localized to the apical surface. One hypothesis is that PGR-A is being exported from the LE into the lumen. Indeed, between days 10 and 14 a large amount of calcium, E2, PGE2 and other proteins are secreted by the uterus into the lumen (Geisert et al, 1982a), stimulated by the high circulating concentration of P4 from the corpus luteum (Chen et al., 1975). The PGR-A protein may be among these. The direct evidence for and implications of the exportation of the PGR-A isoform to the uterine lumen remain to be investigated.

CHAPTER VI

CONCLUSIONS AND DIRECTIONS FOR FURTHER STUDY

CONCLUSIONS

This project focused on the identification and characterization of PGR isoform expression at the mRNA and protein levels in cyclic and early pregnant porcine endometrium, with additional subcharacterization of cell-type specific protein isoform expression within the endometrium.

The first specific aim of the project was to obtain the mRNA and amino acid sequences of porcine PGR, perform *in silica* analysis of gene and protein conservation between similar species and identify sequence characteristics. The PGR mRNA sequence has high homology with human, mouse and cow PGR mRNA (84%, 79% and 86%, respectively). The full-length PGR mRNA isoform is 4.3 kb in pigs. Three start codons give rise to 3 in-frame open reading frames, resulting in 3 potential protein isoforms with progressive N-terminus truncations (PGR-B, PGR-A and PGR-C). Pig PGR protein sequence was conserved in human, mouse and predicted cow PGR sequences (84%, 80% and 72%, respectively) with greater conservation in the functional domains (DBD, hinge, LBD) than the variable region, as anticipated. Less variability is tolerated in the C, D, and E domains than in the A/B domain because they must function efficiently and with

high specificity. Sequence polymorphisms and their consequences on protein sequence were identified.

The second specific aim was to develop a method for the detection of porcine PGR mRNA isoforms in the pig uterus and characterize the expression of these isoforms at different time points during the estrous cycle and in early pregnancy. Day of the cycle or pregnancy affected the mRNA expression of PGR isoforms. Expression of PGR-B isoform mRNA was abundant on d 0 and d 5 and was decreased on d 7.5 in cyclic and d 15 in both cyclic and pregnant pigs. Expression of PGR-AB mRNA (measuring both the PGR-B and PGR-A transcripts) was low and constitutive on all days of pregnancy and the cycle except on d 15 when a transient surge in transcript abundance occurred. The d 15 fold change induction of PGR-AB mRNA was nearly doubled in pregnant pigs versus cyclic pigs. There is evidence for the existence of additional transcripts in porcine endometrial mRNA using qRT-PCR. Expression of PGR-ABC mRNA (measuring PGR-B, PGR-A and PBR-C transcripts) was abundant on d 0 and d 5 and was decreased on d 7.5 and d 12, with the other time points measured intermediate. However, since a distinct PGR-C transcript was not apparent in Northern blot analyses (Chapter 4), qRT-PCR may be detecting the presence of degraded transcripts and not a third PGR isoform (PGR-C).

The transcription initiation sites for PGR were mapped using RPA. There exist two transcription initiation sites for PGR-B (*PGR-B.1* and *PGR-B.2*). The *PGR-B.1* maps to bases 291 – 314, and *PGR-B.2* is located at base 371 (GQ903679.1). Both encode the entire PGR-B protein isoform. The observation was consistent with sequence amplification data in Chapter 2, indicating that a transcription initiation site was located between bases 1 and 470 of GQ903679.1 sequence. The transcription initiation site for

PGR-A is located at nucleotide 1050 (GQ903679.1) and encodes the full PGR-A protein isoform. Finally, there are 2 transcription initiation sites downstream of the PGR-A translation start codon at 2559 and 2633 bp that appear to encode PGR-C (GQ903679.1). The PGR-C isoform could not be observed on any of the Northern blots performed, however. The results from the qRT-PCR experiment would be concordant with the RPA data in suggesting that a PGR-C transcript does exist in porcine endometrium. It is possible that the PGR-C transcript may be unstable and is degraded by the Northern blot technique, impeding the ability of the technique to detect this isoform.

The third specific aim of the project was to confirm the presence of PGR isoform protein variants and then to detect and characterize PGR isoform expression at the protein level at different stages of the estrous cycle, both in total endometrium and in individual cell types. In the porcine endometrium, PGR-B (120 kDa) and PGR-A (96 kDa) protein isoforms can be detected by Western Blot and localized by IHC. Modification of PGR by the protein SUMO may be responsible for the discrepancy between predicted molecular weight and the molecular weight to which PGR-B and PGR-A migrate by electrophoresis. Concentration of isoform abundance remained constant over the days analyzed (d 0, d 8, d 12), with PGR-B more abundant than PGR-A.

Nuclear staining of PGR-B was observed in the LE and GE of d 8 of porcine endometrium and decreased on d 12, as detected by IHC. On both d 8 and d 12, the PGR-A isoform was expressed in the cytoplasm of LE and GE and intermittently in the nucleus of stroma cells. No nuclear staining was observed with PGR-AB antibodies in the LE, suggesting that these antibodies were unable to detect PGR-B in its native conformation. Localization of PGR-B to the nuclei and PGR-A to the cytoplasm may

indicate that on d 8 and d 12 of the estrous cycle, PGR-B may be functionally active as a transcription factor (albeit down-regulated from d 8 to d 12) while PGR-A may be inactive or targeted for degradation. Cytoplasmic PGR-A protein is localized specifically to the apical surface of the LE, which may indicate exportation of the protein into the uterine lumen.

The project, therefore, succeeded in addressing the central hypothesis of the study that PGR mRNA and protein isoforms exhibit different patterns of expression throughout the phases of the estrous cycle and during early pregnancy in the porcine endometrium. We conclude that porcine PGR has high nucleotide and amino acid conservation with human, mouse and cow sequences. We conclude that abundance of mRNA and abundance of protein are, at times, uncoupled in this system, with PGR-C present as an mRNA transcript but not a translated protein. Abundance of mRNA and abundance of protein, at other times, appear to act in tandem, with PGR-B mRNA and PGR-B protein nuclear staining in the LE and GE both decreasing from d 8 – d 12. We also identified new questions to be investigated to increase understanding of PGR isoform expression in the porcine endometrium.

DIRECTIONS FOR FURTHER STUDY

Sequencing of PGR mRNA in additional pigs with diverse genetic backgrounds (different breeds) should be done to identify additional SNPs segregating within the

porcine genome and to validate the SNPs discovered here. The SNPs in PGR may be analyzed as quantitative trait loci affecting fertility traits. A multi-generational pedigree of pigs with available fertility expected progeny differences (EPDs), such as number born alive (NBA), should be compiled and samples acquired. These animals should then be genotyped for the identified SNPs and subjected association analyses to determine correlation of these SNPs with fertility traits. Additionally, attempts should continue to amplify full-length (4.3 kb) PGR from cDNA to ensure accuracy of the mRNA sequence and produce a full-length PGR clone for cataloguing.

Future studies should investigate the cellular localization of PGR-B, PGR-A and PGR-C mRNA in cyclic and pregnant porcine endometrium. Better understanding of cell-specific expression of PGR mRNA isoforms may help to further elucidate patterns of isoform expression concentrations. *In situ* hybridization would be a useful technique to address the cellular location of PGR mRNA isoforms within the endometrium. The localization of PGR isoform mRNA in LE, GE and stroma and also the location of the transcripts within individual cells may facilitate inferences about the stability and viability of these transcripts.

Real-time PCR should also be used on individual cell types (LE and stroma) to avoid dilution of expression changes in mRNA because samples contain a heterogeneous mixture of cells. A homogeneous sample of cells may more clearly demonstrate the anticipated expression of PGR-B, decreasing from d 0 and 5 to barely detectable on d 10 and recovering by d 17. Since the qRT-PCR primer set for PGR-AB detects both PGR-B and PGR-A isoforms, a clearer pattern of PGR-B expression during the cycle would simplify elucidation of the cumulative PGR-AB results.

Detection of PGR-C mRNA presence outside of the uterus should be analyzed by Northern blots to decrease the probability that the PGR-C isoform detected in an artifact of tissue handling. Individual cell-type mRNA extraction and analysis by Northern blot could also be used to determine PGR presence and expression patterns in individual endometrial cell types (LE and stroma). A homogeneous sample of cells may clarify the pattern of changes in PGR-B, PGR-A, and PGR-C isoform mRNA abundance.

Future studies should investigate the presence of PGR-B and PGR-A proteins at additional time points during the estrous cycle and pregnancy. Additionally, the presence of the PGR-C protein should be investigated in other porcine tissues to minimize the probability that the absence of this isoform is tissue-specific, before concluding that the PGR-C isoform is not translated into a protein. This may help to further elucidate patterns of expression concentration and localization. Protein studies should be conducted on endometrium tissue from pigs in early pregnancy to determine any similarities or differences between patterns of PGR isoform expression concentration or localization from cyclic animals.

The function of PGR protein was not addressed by this study. Just as the presence of mRNA does not necessarily indicate the presence of protein, the presence of protein does not necessarily indicate the functionality of the protein. To address this question, several binding assays should be performed, as described (Diekman and Anderson, 1983; Geisert el al., 1994; Beum-Soo et al., 2006; Connaghan-Jones et al., 2007). First, LE and stroma cells should be incubated with radiolabeled P4 to determine the total capacity of the PGR present in the cells to bind its ligand. Additionally, saturation analysis, adding increasing amounts of radiolabeled P4 to LE and stroma cells and measuring the unbound

fraction, should be conducted to determine the dissociation constant and number of functional receptors present in the cells (Radcliff et al, 2003). Ilenchuk and Walters developed a method to assay isoform – specific function by photoaffinity labeling (Ilenchuk and Walters, 1987). To validate isoform – specific function, qRT-PCR should be employed to assay the mRNA abundance of known targets of PGR-B – or PGR-A – stimulated transcription in homogeneous tissue samples (Tung et al., 2006; Jacobsen et al., 2005).

The presence of SUMO needs to be confirmed directly, using a deconjugating enzyme and/or coimmunoprecipitation of PGR and SUMO. SUMO is deconjugated both in vivo and in vitro by enzymes called SENPs (sentrin/SUMO-specific proteases) that break the covalent bond between SUMO and the glycine residue of the target protein (Colby et al., 2006). Once SUMO presence is validated, the amount of SUMOylation of the PGR isoforms in cells can be used to determine whether amount of SUMOylation can be correlated to PGR-B and PGR-A activity or cellular localization. SUMO presence would also account for the discrepancy between expected molecular weight of PGR-B and PGR-A isoforms and the molecular weight to which the isoforms migrate on a gel in human (Abdel-Hafiz et al., 2002; Jones et al., 2006; Chauchereau et al., 2003) and pig (Slomczynska et al., 2000; Shimada et al., 2004).

Additional elucidation of PGR isoforms at both the mRNA and protein levels will lead to a more complete understanding of the mechanisms controlling the estrous cycle and pregnancy. A more thorough understanding of the process opens the door to better solutions to prevent and treat aberrant estrous cyclicity and embryonic loss in pigs. Due to the high homology of transcripts and proteins, results may even be applicable to

similar species, including cattle, mice and maybe humans which all have high homology to pig PGR at the mRNA and protein levels.

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VITA

Erin Sellner was born in New Ulm, Minnesota on May 25th, 1982. She received her junior high education from New Ulm Area Catholic Schools. Her family then relocated to Jefferson City, Missouri and she graduated from Jefferson City High School in May, 2000. During an Honors Biology course at JCHS, Erin became engrossed in genetics and the concept of inheritance.

She was accepted to the University of Missouri – Columbia Department of Animal Science, and began coursework for a Bachelor's Degree in August of 2000. During her coursework, she became interested in the prestigious F21C Reproductive Biology group at MU. In December of 2003, Erin received her Bachelor's Degree in Animal Science with dual minors in Rural Sociology and English.

Erin then spent a semester evaluating graduate schools and working as a laboratory technician in the lab of Dr. Jeremy Taylor. In June 2004, she joined Dr. Taylor's lab as a doctoral student in functional genomics. She transferred to the lab of Dr. Matthew Lucy in January of 2007 to continue her doctoral research with emphases in both genetics and reproductive physiology. Erin has been studying the expression of different isoforms of progesterone receptor in the uterus of pigs, under the supervision of Dr. Lucy. She has also completed requirements to receive a doctoral minor in college teaching. During her tenure as a graduate student, Erin has served as chairperson of a student-organized genetics journal club, served as chairperson of several committees of the Animal Science Graduate Student Association, and developed a graduate-level course

on the female experience of a career in Animal Science. Erin is interested in pursuing a teaching-intensive career in Animal Science and exploring opportunities to pursue scientific writing.