A novel first exon directs hormone-sensitive transcription of the pig prolactin receptor

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Abstract

Endocrine, paracrine, and autocrine prolactin (PRL) acts through its receptor (PRLR) to confer a wide range of biological functions, including its established role during lactation. We have identified a novel first exon of the porcine PRLR that gives rise to three different mRNA transcripts. Transcription of this first exon is tissue specific, where it increases during gestation in the adrenal glands and uterus. Within the mammary glands, its transcription is induced by estrogen and PRL, while in the uterus, its expression is downregulated by progestin. The promoter region has an enhancer element located between −453 and −424 bp and a putative repressor element between −648 and −596 bp. Estrogen, acting through the estrogen receptor, activates transcription from this promoter through both E-box and transcription factor AP-2 α binding sites. These findings support the concept that the multilevel hormonal regulation of PRLR transcription contributes to the various biological functions of PRL.

Key Words
- promoter
- prolactin receptor
- alternative splicing
- pig

Introduction

Prolactin (PRL) is a protein hormone that has diverse biological functions including pronounced roles in adaptive stress responses, osmoregulation, uterine function, proliferation and differentiation of the mammary glands, and lactation (Bole-Feysot et al. 1998). PRL functions through multiple forms of its membrane-associated receptor (PRLR) that differ primarily within the intracellular domain as described for human, mouse, rat, cow, pig, sheep, goat, and carp. Upon ligand activation, these various isoforms can differentially activate downstream signal transduction pathways (reviewed in Trott et al. (2012)). The PRLR is expressed in various tissues and cell types, where its levels vary during states including the estrus cycle, pregnancy, and lactation (Jahn et al. 1991, Hovey et al. 2001, Trott et al. 2009). The PRLR is also expressed in a majority of human breast cancers (Gill et al. 2001, Peirce & Chen 2001), where antagonism of the PRLR potentiates the cytotoxic action of anti-cancer drugs and reduces PRL-induced cell survival (Howell et al. 2008).

Expression of the PRLR across various tissues and physiological states is under complex transcriptional regulation. Six alternative non-coding first exons have been reported in humans, including a generic exon 1 (hE13) that is orthologous to E13 in rat and mouse (Hu et al. 1999, 2002). The E13 is expressed in the mammary glands, ovaries, Leydig cells, and liver of both humans and rats (Moldrup et al. 1996, Hu et al. 1999). In addition to E13, four other gonad-, liver-, and brain-specific first exons have been identified in rats (Hu et al. 1996, Moldrup et al. 1996, Hu et al. 1997, Tanaka et al. 2002, 2005) and mice.
(Ormandy et al. 1998, Tabata et al. 2012). Transcription from these alternative first exons is controlled by upstream promoters that recruit specific cis-acting factors. In turn, activation of these promoters could lead to differential expression of the PRLR across tissues, thereby contributing to the diverse biological functions of PRL.

Apart from data for humans, rats, and mice, no literature is available on the 5′ UTR of the PRLR or its regulation in livestock where reproduction and lactation underlie a multi-billion dollar industry. At the same time, production animals are increasingly important as biomedical models for cancer, obesity, and infertility (Kuzmuk & Schook 2011). Furthermore, expression of medical models for cancer, obesity, and infertility production animals are increasingly important as bio-

regulation in livestock where reproduction and lactation control been investigated for established PRL target tissues such as the mammary glands or uterus in vivo.

In this study, we identified multiple splice variants of a porcine PRLR E1 (pE1). We investigated the expression of three pE1 splice variants across various tissues in virgin and pregnant females and found that expression levels increased during pregnancy in the adrenal glands and endometrium. Expression analysis in a ‘hormone-replacement’ model in vivo established that pE1 was positively regulated by β-estradiol (E) and PRL in the mammary glands and negatively regulated by progestin in the uterus. Furthermore, E activated the pE1 promoter in E-responsive breast cancer cells through E-box and transcription factor AP-2 α (TFAP2A) binding sites.

Materials and methods

Animals

Virgin and pregnant (day 105 of gestation) crossbred (Hampshire × Yorkshire) gilts and boars were housed, treated, killed, and their tissues sampled as described (Trott et al. 2011). Tissues from pregnant (day 105 of gestation) gilts, and testis from a boar, were used for 5′ RACE. Tissues from virgin gilts were used to determine tissue-specific expression in non-pregnant animals. Pregnant, unilaterally hysterectomy-ovariectomized gilts were killed at day 25, 45, 65, 85, or 105 of gestation and were housed, treated, and sampled as described (Freking et al. 2007). Hormone-treated peripubertal Yucatan miniature pigs were housed, treated, killed, and their tissues harvested as described (Horigan et al. 2009). A total of 36 gilts were used, of which four were sham-operated, while 32 were ovariectomized. One day after surgery, ovariectomized gilts began receiving bromocriptine (Br; 0.1 mg/kg per day i.m.) for 8 days to suppress PRL secretion. Beginning on day 9, gilts were injected i.m. with various combinations of E (0.1 mg/kg per day), medroxyprogesterone 17-acetate (a synthetic progestin (P), 0.25 mg/kg per day), and/or Br or haloperidol (Hal; 1.5 mg/kg per day; to induce endogenous PRL release) for 5 days. The ovariectomized, Br-treated gilts were assigned to one of the eight treatments (n = 4 per group): Br, Br + E, Br + P, Br + E + P, Hal, Hal + E, Hal + P, and Hal + E + P. Sham-ovariectomized gilts received daily injections of saline for 14 days. All gilts were killed 14 days after surgery.

5′ RACE

Poly A + RNA (Oligotex mRNA Mini Kit, Qiagen) was isolated from mammary gland, placenta, liver, kidney, adrenal, ovary, and testis, and 5′ RACE was performed using the SMARTer RACE cDNA amplification kit (Clontech) with two gene-specific primers: GSP1a, 5′-CGGAGGTGACTGTCCATTCAGAAGGCTG-3′ and NSGSP1a, 5′-TTGGCTCCCTTCTCTTTCACAGGCAG-3′ (designed from the pPRLR cDNA, GenBank ID: NM_001001868) or the 5′/3′ RACE Kit (Roche) with primers reported in Trott et al. (2007) using two rounds of nested PCR. 5′ RACE products from the nested PCR were identified by electrophoresis on 1.2% agarose gels, gel-purified, and cloned into pCR2.1-TOPO (Invitrogen). Clones were analyzed by restriction enzyme digestion and agarose gel electrophoresis before sequencing. The sequence of the pPRLR first exon was confirmed by sequencing a bacterial artificial chromosome (BAC) containing the pPRLR (CHORI-242 255C20, a gift from Dan Nonneman, USDA-ARS) using a sequencing primer in the newly discovered pE1.

RNA extraction and RT

Tissues were homogenized in TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and total RNA was extracted according to the manufacturer’s instructions. Total RNA (5 μg) was treated with DNaseI (Roche) and purified using the DNA-Free RNA Kit (Zymo Research Corporation, Orange, CA, USA) before confirmation of its integrity by formaldehyde-agarose gel electrophoresis. Total RNA (500 ng) was denatured at 70°C for 5 min along with oligo-dT (50 ng, Amersham Biosciences) and random hexamers (2 ng, Amersham Biosciences) and then reverse transcribed using 1×RT
Buffer (Promega), dNTP mixture (0.016 mM, Promega), Moloney’s murine leukemia virus reverse transcriptase (2 U/μl, Promega), and RNase inhibitor (0.85 U/μl, Promega) at 37 °C for 90 min, followed by denaturation at 95 °C for 5 min.

Cloning pE1 transcripts

Mammary gland cDNA was amplified with GoTaq Green Master Mix (Promega) using 0.2 μM each of the primers pE1_F, 5'-GACTGATACGTGACACTGACTTT-3' and pE3/4_R, 5'-GGAGGTGACTGTCCATTCAGA-3' (spanning exons 3 and 4) to confirm the existence of splice variants involving the first three exons of the pPRLR. Different-sized PCR products were agarose gel-purified, cloned into pCR2.1-TOPO, and sequenced.

Quantitative PCR

The cDNA was diluted 1:1, and 4 μl was used as a template for real-time quantitative PCR (qPCR) containing Fast SYBR Green Master Mix (Applied Biosystems) and 0.2 μM of each primer on a 7500 Fast Real-Time PCR System (Applied Biosystems), using primers named for the exons in which they are located: pE1/3_F, 5'-CCGGGCAAAT-GAACCTCTGA-3' and pE3, 5'-TGTTGAGTGCCACA-TTTTCTT-3'; pE1_F, 5'-GACTGATACGTGACACTGACTTTGCTTT-3' and pE2/3_R, 5'-GGTGGAGTGCTCATTACAAGGTTTTGA-3'; pE1_F, 5'-GACTGATACGTGAGCTGACAGTGGTCAAAGGCTGTCA-3'; pE1_F, 5'-GACTGATACGTGACACTGAGTGGTCAAAGGCTGTCA-3'; pE2/2.1_R, 5'-GAATGGGAGCCCTCAGGG-3', for transcripts pE1/3, pE1/2/3, and pE1/2/2.1/3 respectively. Samples were analyzed as duplicates. A standard curve with a range from 10 to 10⁷ copies was prepared for each pPRLR transcript using mouse liver cDNA spiked with the respective number of copies of pPRLR cDNA cloned into pCR2.1-TOPO. The standard curve was spiked into mouse cDNA to ensure that the standard curve and samples had a similar composition.

Table 1: Primers used for PCR amplification of pPRLR E1 promoter fragments cloned into pCR2.1

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Primer sequence (5'-3')</th>
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<tbody>
<tr>
<td>pPRLRE1_F170</td>
<td>CCAACCGAGAACACAGAAAAGTA</td>
</tr>
<tr>
<td>pPRLRE1_F424</td>
<td>CTTGGGTCCAGCAGAAATAT</td>
</tr>
<tr>
<td>pPRLRE1_F651</td>
<td>CTTGGAAATTTTTGGGTTTTA</td>
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<tr>
<td>pPRLRE1_F859</td>
<td>TGTGTTGTTGTTGGTTTT</td>
</tr>
<tr>
<td>pPRLRE1_F1002</td>
<td>CCGTCTGGTTAGAAGAAAAGG</td>
</tr>
<tr>
<td>pPRLRE1_F1176</td>
<td>AGTTCCCTGGGATACGCAGT</td>
</tr>
<tr>
<td>pPRLRE1_R</td>
<td>CTGCAAAAGGACGTGAGA</td>
</tr>
</tbody>
</table>

The same standard curve was used in duplicate or triplicate across multiple plates for each transcript. Standard curves were generated by linear regression of Ct vs log₁₀ (dilution factor). Melting curve analysis was used to confirm the specificity of PCR products, and a minus-RT control was used to ensure that products were from cDNA rather than genomic DNA. Gene expression levels were normalized for 18S rRNA expression (Trott et al. 2011).

Construction of promoter plasmids

A 1171-nucleotide promoter sequence was obtained by sequencing BAC CHORI-242 255C20 using a reverse primer in pE1 and primer walking upstream. Promoter fragments of various lengths were generated by PCR from this BAC by one of the two methods. First, fragments were generated using primers listed in Table 1 and Extender PCR mix (ABgene, Epsom, UK) followed by subcloning into pCR2.1-TOPO. Promoter fragments were then excised and ligated into the promoterless pGL3-Basic vector (Promega) using the Quick Ligation Kit (New England BioLabs, Ipswich, MA, USA). Further deletions of the pE1 promoter were generated by PCR using primers listed in Table 2 and GoTaq Green Mastermix (Promega). These fragments were ligated into pGL3-basic using the In-Fusion PCR Cloning system (Clontech). Mutations in the TFAP2A and E-box binding sites of the 201 and 242 bp pE1 promoter were generated by PCR using primers listed in Table 3 and Extender PCR mix (ABgene, Epsom, UK) followed by subcloning into pCR2.1-TOPO. PCR was performed using GoTaq Green Mastermix (Promega) and products were ligated into pGL3-basic using the Gibson Assembly

Table 2: Primers used for PCR amplification of pPRLR E1 promoter fragments cloned using the In-Fusion PCR cloning system

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Primer sequence (5'-3')</th>
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<tbody>
<tr>
<td>pPRLRE1_201F</td>
<td>TATCGATAGGTACCGTGAGCTTAAGAATCCA</td>
</tr>
<tr>
<td>pPRLRE1_242F</td>
<td>TATCGATAGGTACCGTCCTCCCTCCTACCTGTTGGT</td>
</tr>
<tr>
<td>pPRLRE1_293F</td>
<td>TATCGATAGGTACCGGGGAAATCCGGTGCTCTTCTTCTT</td>
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<td>pPRLRE1_353F</td>
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<td>pPRLRE1_453F</td>
<td>TATCGATAGGTACCGGTTTCTTGCTCGTCTCGGTGAGTACG</td>
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<td>pPRLRE1_500F</td>
<td>TATCGATAGGTACCGGTTTCTTGCTCGTCTCGGTGAGTACG</td>
</tr>
<tr>
<td>pPRLRE1_567F</td>
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</tr>
<tr>
<td>pPRLRE1_596F</td>
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</tr>
<tr>
<td>pPRLRE1_infR</td>
<td>TATCGATAGGTACCGGTTTCTTGCTCGTCTCGGTGAGTACG</td>
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Transient transfection of MCF-7 cells and reporter gene assays

Human MCF-7 breast cancer cells were cultured in DMEM supplemented with 10% FBS, 100 U penicillin and 100 µg streptomycin/ml, and 2.42 g/l HEPES. Cells were plated into 12-well plates at 2 × 10^5 cells/well, then 1 day later were transiently transfected with 0.8 µg of either pE1 promoter-pGL3 plasmid or pGL3-basic DNA and 0.2 µg of either pEF-lacZ or pCMV-eGFP plasmid using 3 µl FuGene6 (Roche). In some studies, cells were transfected with 0.4 µg of either pE1 promoter-pGL3 plasmid or pGL3-basic plasmid and 0.1 µg of pCMV-eGFP plasmid using 3 µl FuGene6 (Roche). In some studies, cells were transfected with 0.4 µg of either pE1 promoter-pGL3 plasmid or pGL3-basic plasmid and 0.1 µg of pCMV-eGFP, using 1.5 µl Lipofectamine LTX and 0.5 µl PLUS (Invitrogen). In the promoter deletion experiment, cells were cultured in complete growth medium and harvested after 24 h. In experiments examining induction of the pE1 promoter by E, the culture medium was changed to hormone-deficient medium (containing 10% charcoal-stripped FBS; Omega Scientific, Tarzana, CA, USA) the day after transfection. Cells were then exposed to E (1 nM) or ethanol vehicle for 48 h. In the experiment using the estrogen receptor (ESR) antagonist ICI 182 780 (ICI; Ascent Scientific, Cambridge, MA, USA; 100 nM in ethanol), the culture medium was changed to hormone-deficient medium the day after transfection. Cells were then exposed to either ICI (100 nM) or vehicle for 24 h before treatment with either E, E+ICI, or vehicle for 48 h in hormone-deficient medium. Cells were lysed with 200 µl Glo Lysis buffer (Promega) and lysates were assayed for luciferase and either β-galactosidase or eGFP activity. Luciferase activities were corrected for transfection efficiency as determined by the relative level of β-galactosidase or fluorescence in each lysate.

Table 3 Primers used for PCR amplification of mutant transcription factor binding sites in the pPRLR E1 promoter, cloned using the Gibson Assembly cloning kit. Altered nucleotides are lower case. The E-box (242 bp promoter) and TFAP2A (201 bp promoter) sites are underlined

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Primer sequence (5’-3’)</th>
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<tbody>
<tr>
<td>pPRLRE1_201mut</td>
<td>CGATAGTGACGAATTGCGACGCCTGAAGAAATCAGTTTatTCC</td>
</tr>
<tr>
<td>pPRLRE1_242mut</td>
<td>CGATAGTGACGAATTGCGACGCCTGAAGAAATCAGCTCCTGC</td>
</tr>
<tr>
<td>pPRLRE1_gibR</td>
<td>AGATCTGACGGCCTGCAGAAAAAGGACGTGGGAGAT</td>
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cloning kit (New England BioLabs). All promoter fragments were sequence-verified.

Transcription factor binding sites

Putative binding sites for transcription factors in the pE1 promoter sequence were identified using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess) and AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). For TESS, only sites with a Log-likelihood score (La) ≥ 10 were considered.

Statistical analysis

Data were analyzed using the General Linear Models procedure in SAS (SAS version 9.3, SAS Institute, Cary, NC, USA). Expression data were log_{10} or power transformed for normalization where appropriate. Expression data from virgin, hormone-treated gilts were analyzed within each tissue by ANOVA followed by Tukey’s post hoc test. Main effects of hormones and their interaction were tested by factorial ANOVA. Expression data from pregnant gilts were analyzed within tissue by linear regression. Luciferase activity data from the promoter deletion and mutation studies were analyzed by ANOVA. Significance was declared at P < 0.05.

Results

Identification of a novel pPRLR first exon

Using 5’ RACE, we obtained 35 clones of 200–250 bp from reverse-transcribed RNA from mammary gland, liver, placenta, adrenal gland, kidney, ovary, and testis. All clones were identified as spanning from a novel exon 1 (pE1) to exon 3 of the pPRLR, where 54% of the clones also contained a 62 bp non-coding exon 2. One clone contained 162 bp of pE1, as confirmed by its alignment with genomic sequence. The 162 bp of pE1 is located more than 91 kb upstream of exon 2 on Sus scrofa chromosome 16 (SSC16; Fig. 1A). Among the clones, 23% had a transcription start site 23 bp downstream that conferred a length of 139 bp for pE1, while 15% of clones had a transcription start 59 bp downstream that conferred a pE1 length of 80 bp. Using RT-PCR, we further identified three distinct splice variants involving exon pE1. Two were identical to our 5’ RACE products, namely pE1/3 and pE1/2/3. The largest PCR product contained a novel exon of 113 bp that is located between exons 2 and 3, which was designated as non-coding exon 2.1 (Fig. 1A), giving rise to the transcript pE1/2/2.1/3. A multiple sequence alignment revealed that pE1 is homologous to the generic E13 in humans, rats, and mice (Fig. 1B).
Tissue-specific expression of pPRLR E1 in virgin and pregnant gilts

Expression of these three pE1 transcripts in a wide range of tissues from virgin gilts was determined by qPCR. The pE1/3 and pE1/2/3 transcripts (\(\sim 10^5\) copies/\(\mu\)g RNA) were most abundant in the adrenal glands, endometrium, mammary glands, pituitary, and placenta (Fig. 2A and B), while the pE1/2/2.1/3 transcript was most abundant in the heart, mammary glands, pituitary, and placenta (Fig. 2C). Very low or no expression of pE1 was detected in liver, lymph node, pancreas, skeletal muscle, and thymus. Notably, expression of the pE1/2/2.1/3 transcript was \(\sim 100\)-fold lower than that for the two other pE1 transcripts in all tissues.

Given the crucial role for PRL during gestation, we further examined expression of the pE1 transcripts in...
Hormonal regulation of pPRLR E1

Given our previous finding that expression of the long form of the pPRLR (pPRLR-LF) is regulated by PRL in the mammary glands, and by E and P in the uterus (Trott et al. 2009), alongside findings by others that E regulates E1 expression in rats and human breast cancer cells, we further examined the regulation of pE1 transcription by E, P, and PRL in the uterus, liver, and mammary glands in vivo (Fig. 4). The female pigs were hormone deficient before treatment with either Br (hypoprolactinemic) or Hal (hyperprolactinemic) in the presence or absence of E and/or P. There was a negative main effect of P on the expression of all pE1 transcripts in the uterus (P<0.0001). For the pE1/3 transcript, there was also a positive main effect of E (P<0.04) and a negative interaction between the effects of E and P (P=0.02), and PRL and P (P=0.05). For the liver, there was a small but significant negative main effect of P on the expression of all three transcripts (P=0.01, 0.01, and 0.04 for pE1/3, pE1/2/3, and pE1/2/2.1/3 respectively), as well as a positive main effect of PRL on the expression of pE1/3 (P=0.01) and pE1/2/3 (P=0.002). Both E and Hal exerted positive main effects (P<0.01) on expression of all three pE1 transcripts in the mammary glands, and there was an interaction between the effects of PRL and P (P=0.01) for expression of the pE1/3 transcript.

Analysis of the putative pPRL R E1 promoter

The 1.2 kb region upstream of pE1 was sequenced from BAC clone CHORI-242 255C20 (Fig. 1C). The promoter region is numbered from the transcription start site defined by the longest 5’ RACE transcript of pE1 (162 bp). We did not identify a TATA box within 40 bp upstream of this, or the other two, potential transcription start sites. We used MCF-7 epithelial breast cancer cells, a widely used model for trans-species E-responsiveness in vitro, to study pE1 promoter activity. Deletion of the region −1171 to −648 bp of the pE1 promoter did not affect its transcriptional activity in transiently transfected MCF-7 cells.

Figure 2
Expression of different pPRLR exon 1 transcripts across various tissues in virgin gilts. Number of pPRLR mRNA copies was measured by qPCR with normalization for the corresponding level of 18S rRNA. (A) Transcript pE1/3, (B) transcript pE1/2/3, (C) transcript pE1/2/2.1/3. Endom, endometrium; Hypoth, hypothalamus; MG, mammary gland; Lymph, lymph node; Sk musc, skeletal muscle; Sm int, small intestine. Data are means ± S.E.M. (n = 3).
Figure 3
Expression of the different pPRLR exon 1 transcripts across tissues from gilts at different stages of gestation. Copy number of total pPRLR mRNA was measured by qPCR with normalization for the corresponding level of 18S rRNA. (A) Transcript pE1/3. (B) Transcript pE1/2/3. (C) Transcript pE1/2/2.1/3.

Endom, endometrium; Hypoth, hypothalamus; MG, mammary gland. Data are means ± s.e.m. (n = 4–5). Where a significant linear relation existed between expression and time of gestation, the $R^2$ for the correlation is shown.
cells. Deleting the region $-648$ to $-424$ bp increased promoter activity by 130%, suggesting the likely presence of a repressor site (Fig. 5A). Further deleting the region $-424$ to $-170$ bp reduced transcriptional activity to that of the promoterless construct. We further analyzed the region $-651$ to $-170$ bp to identify putative cis elements that might regulate expression from the pE1 promoter (Fig. 5B). The aforementioned putative repressor site was
Regulation of PRLR expression in humans, rats, and mice are mediated by Sp1 binding sites that were previously shown to be involved in C/EBP and specificity protein 1 (Sp1) binding sites. We identified a putative TFAP2A binding site between 645 to 648 bp and a putative E-box sequence (5'-CANNTG-3') between 596 to 594 bp. Localization of the basal transcription factor binding sites is required for E13 basal activity in rats (Hu et al. 1998). We examined the putative TFAP2A site in the pE1 promoter and found that the magnitude of E-activation from the 242 region was higher than from the 201 fragment. We also identified a putative TFAP2A binding site between 166 and 174 bp (consensus G/C C C N N A/C/G G/A G G/C/T; McPherson & Weigel 1999) and an E-box sequence (5’-CANNTG-3’) from 232 to 227 bp. We generated two constructs that contained either a mutated TFAP2A site or a mutated E-box in the 201 promoter or a mutated E-box in the 242 promoter. Estrogen-induced activation of the 201 promoter was ablated when the TFAP2A site was mutated (Fig. 6A), while E-activation of the 242 promoter was ablated by mutation of the E-box (Fig. 6B). The basal transcription levels were also reduced by mutating both the TFAP2A site and the E-box (Fig. 6A and B). Given that the E-box and TFAP2A sites are not classical EREs, we used the ESR antagonist ICI 182 780 and confirmed that the ESR is involved in the E-induced activation of these 201 and 242 bp pE1 promoter fragments (Fig. 7).

Discussion

We find that the pE1 is structurally and functionally similar to the hE13, rE13, and mE13 with regard to sequence homology, conserved cis-regulatory elements, and level of expression across tissues. These first exons in other species are regarded as the principal first exon for the PRLR gene. Furthermore, we identified that exon 2 is a variably spliced region of the pE1 promoter that is inactive in MCF-7 cells.

Using TESS and Alibaba, we also predicted transcription factor binding sites. We identified a putative TFAP2A binding site between 166 and 174 bp (consensus G/C C C N N A/C/G G/A G G/C/T; McPherson & Weigel 1999) and an E-box sequence (5’-CANNTG-3’) from 232 to 227 bp. We generated two constructs that contained either a mutated TFAP2A site in the 201 promoter or a mutated E-box in the 242 promoter. Estrogen-induced activation of the 201 promoter was ablated when the TFAP2A site was mutated (Fig. 6A), while E-activation of the 242 promoter was ablated by mutation of the E-box (Fig. 6B). The basal transcription levels were also reduced by mutating both the TFAP2A site and the E-box (Fig. 6A and B). Given that the E-box and TFAP2A sites are not classical EREs, we used the ESR antagonist ICI 182 780 and confirmed that the ESR is involved in the E-induced activation of these 201 and 242 bp pE1 promoter fragments (Fig. 7).
constituent of transcripts arising from pE1. Splicing of exons 1–3, while omitting exon 2, has also been reported for both the rPRLR (Hu et al. 1996) and the hPRLR (Hu et al. 1999). To our knowledge, the existence of an exon 2.1 has not been reported in any other species, although a 70% homologous sequence is present within the intron between E2 and E3 in the hPRLR gene. Alternative splicing of exon 2 and/or 2.1 would further diversify the PRLR 5' UTR by deleting regulatory cis elements. This could affect PRLR mRNA stability or the rate of protein translation.

Among the various tissues we examined, expression of pE1 was most abundant in PRL-sensitive tissues and increased during gestation, similar to the expression profile for the pPRLR-LF (Trott et al. 2009). Previous analysis of the hormonal regulation of E1 transcription in vivo have been limited to select tissues such as the liver, pituitary gland and in a subset of brain regions from rats and mice or in murine and human mammary epithelial cell lines in vitro (Fi et al. 2003, Tanaka et al. 2005, Dong et al. 2006, Kobayashi et al. 2007, Goldhar et al. 2011, Tabata et al. 2012). The induction of pE1 expression by E and its repression by P in the uterus of pigs aligns with the effect of these hormones on pPRLR-LF mRNA expression. Alternating concentrations of serum hormones during gestation may account for the changes in pE1 expression during this period (Eldridge-White et al. 1989). Here, we show that E increases pE1 mRNA expression in the mammary glands of pigs, consistent with our previous finding that E specifically induces pPRLR-LF expression in the mammary epithelium. It is noteworthy that the E-induction of pE1 expression here was much higher than that for pPRLR-LF (Trott et al. 2009). These results also align with our findings in breast cancer cells herein and similar reports by others (Dong et al. 2006). Investigations into the effect of progestins on E1 expression have been limited to mice and transiently transfected T47D cells.

**Figure 6**
Analysis of transcription factor mutants for the pE1 promoter in MCF-7 cells transiently transfected with plasmids containing the promoter upstream of a luciferase reporter. Luciferase activity in cells transfected with pGL3-Basic and not stimulated by β-estradiol (E) was set to 1. Cells in hormone-deficient media were treated for 48 h with either 1 nM E or vehicle. (A) The 201 bp promoter was mutated at the TFAP2A binding site (−201mut). Data are means ± S.E.M. (n = 3) from three independent experiments. (B) The 242 bp promoter was mutated at the E-box sequence (−242mut). Data are means ± S.E.M. (n = 3) from three independent experiments. A significant difference between the response to vehicle and E is indicated by *. a,b,cMeans without a common superscript differ (P < 0.05).

**Figure 7**
The β-estradiol (E) receptor antagonist ICI 182 780 (ICI) inhibits E-induced activation of the pE1 promoter in MCF-7 cells transiently transfected with plasmids containing the promoter upstream of a luciferase reporter. Luciferase activity in cells transfected with pGL3-Basic and not stimulated by E was set to 1. Cells were pre-treated for 24 h with 100 nM ICI or vehicle in hormone-deficient media followed by 48 h treatment with vehicle, 1 nM E, or E + ICI. Data are means ± S.E.M. (n = 3) from three independent experiments. A significant difference between the response to vehicle and E is indicated by *. a,b,cMeans without a common superscript differ (P < 0.05).
breast cancer cells where progesterone induced mE13 transcription (Goldhar et al. 2011). Expression of pE1 in the liver is far less sensitive to hormonal regulation compared with the uterus or the mammary glands. This finding is similar to the complete lack of hormonal regulation for pPRLR-LF mRNA expression in the liver (Trott et al. 2009) and the finding that neither gonadectomy nor sex steroid hormone treatment affected E13 expression in the liver of rats (Tanaka et al. 2005). Others have suggested that E regulates eE13 expression in some regions of the brain such as the hypothalamus and the choroid plexus, although discrepancies exist regarding the existence and the direction of this effect (Pi et al. 2003, Kobayashi et al. 2007, Nogami et al. 2007, Tabata et al. 2012). This differential hormonal responsiveness may reflect a varied presence of tissue-specific transcription factors across different stages of development.

While C/EBP and Sp1 sites are critical for E-induced hPRLR expression (Hu et al. 2002, Dong et al. 2006), our results indicate that the proximal C/EBP and Sp1 binding sites located between −151 and −131 are not involved in either the basal promoter activity for pE1 or its responsiveness to E. We find that the sequence between −648 and −170 of the pE1 promoter is the primary regulator of transcription and that two proximal non-ERE but E-sensitive sites, an E-box and an TFAP2A site, contribute to ER-induced transcription directed by the pE1 promoter. Both also contribute to basal transcription. Dong et al. (2006) also failed to identify functional EREs in the homologous hE13 and proposed that transcription resulted from ligand-bound ER recruited to C/EBPβ and Sp1 response elements in the proximal part of the promoter while forming complexes with additional coactivators. We suggest two ways in which E-activation of the ER might activate the E-box to induce transcription from the pE1 promoter. First, E may directly activate upstream stimulator factors (USFs) to bind the E-box or it may activate the ESR to complex with USFs at the E-box. USF-1 binds an imperfect E-box through a complex involving USF1, ESR1, and Sp1 proteins that is absolutely required for transcription from the ESR1 promoter (deGraffenried et al. 2004). Secondly, the ESR can directly activate USFs to bind an E-box in the cathepsin D promoter (Xing & Archer 1998). Meanwhile, it is less clear how E-activation of the ER acts on the putative TFAP2A site to induce pE1 promoter activity. Others showed that TFAP2A is a major regulator of E-signaling in MCF-7 cells (Woodfield et al. 2007) and in ESR-positive breast cancer cells where it regulates transcription of the ER promoter upstream of the P1 cap site (deConinck et al. 1995). However, our discovery that a putative TFAP2A site in the porcine PRLR promoter is required for ESR-induced transcription is the first report of E-activation of a putative TFAP2A site. The region between −648 and −596 bp of the pE1 promoter includes a candidate repressor region that contains putative binding sites for Pit-1 and ETS. Pit-1 is primarily expressed in the pituitary but has also been detected in the extrapituitary tissues such as placenta, lymphoid, and hematopoietic tissues, as well as in the normal and tumor breast, and in MCF-7 cells. This factor is responsible for the transcriptional regulation of GH, PRL, and TSH expression (Holloway et al. 1995, Gil-Puig et al. 2005). ETS factors act as transcriptional repressors and/or activators and can be either ubiquitous or tissue specific (Sharrocks 2001, Wei et al. 2010).

In summary, we have identified species-specific differences in the PRLR E1 promoter that may underlie the functional diversity of PRL across species. The complex multilevel transcriptional control of the PRLR could be crucial to the many biological functions of PRL.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This project was supported by National Research Initiative Competitive grant no. 2008-35206-18895 from the USDA National Institute for Food and Agriculture.

Acknowledgements
The authors thank Kent Parker and interns from the UC Davis Swine Research and Teaching Facility for animal care and Caleb Sehnert from the UC Davis Meat Lab for assistance with tissue collection.

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Received in final form 29 March 2013
Accepted 10 April 2013
Accepted Preprint published online 10 April 2013